Human anti-(bovine milk fat globule membrane) antibodies: involvement in coronary heart disease

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HUMAN ANTI-(BOVINE MILK FAT GLOBULE MEMBRANE) ANTIBODIES: INVOLVEMENT IN CORONARY HEART DISEASE

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for the degree of Ph.D.
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SUMMARY

Davies and coworkers, in 1974, reported that levels of anti-(whole cows' milk) antibodies were significantly raised in patients who had suffered a Myocardial Infarction (MI), compared to controls. In 1982 Davies et al, described that such antibodies were more specifically directed to a component of cows' milk, the milk fat globule membrane (BMFGM). Davies et al (1982), further showed that a possible cross-reaction, between anti-BMFGM antibodies and the platelet membrane may exist, possibly providing a pathogenic mechanism contributing to MI.

The work in this thesis has examined in more detail;

1. Anti-BMFGM antibodies in MI and control patients.
2. The antigenicity of the BMFGM.
3. The cross-reactivity of the BMFGM and the platelet membrane.

Using an ELISA, developed to detect anti-BMFGM antibodies, it was found that IgM anti-BMFGM antibodies were raised in MI patients, compared with controls. IgA and IgG anti-BMFGM levels were also measured, but no significant differences were found between MI and control groups.

A method of affinity purifying anti-BMFGM antibodies was developed, and using these, together with human

sera, as immunochemical probes of the BMFGM, a protein of Mr 150-155kD, corresponding to xanthine oxidase (XO), was shown to be a major antigen of the BMFGM. XO was further purified, and used to detect anti-XO antibodies in serum. A strong correlation was shown between anti-XO levels, and anti-BMFGM levels in individual sera. On examination of anti-XO antibody levels in MI and control sera, significant elevations of IgM anti-XO antibodies were seen in MI sera*, compared to controls, but not with IgG or IgA.

No specific cross-reaction between anti-BMFGM antibodies and the platelet membrane was found. However, it was shown that anti-BMFGM antibodies may bind to platelets in the form of immune complexes.

These findings are discussed with reference to Davies' findings and with reference to the inability of other workers to repeat his findings.

* see Appendix

List of Abbreviations

AEC	3-Amino-9-ethylcarbazole
BMFGM	Bovine milk fat globule membrane
BSA	Bovine serum albumin
CHD	Coronary heart disease
CIC	Circulating immune complex
ConA	Concanavalin A
DMF	Dimethylformamide
DMSO	Dimethysulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EMT	Erythrocyte migration time
Gly	Glycine
HDL	High density lipoprotein
HRP	Horseradish peroxidase
IgA	Immunoglobulin A
s.IgA	Secretory IgA
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ITP	Idiopathic thrombocytopenic purpura
LDL	Low density lipoprotein
MI	Myocardial infarction
MG	Myasthenia Gravis
MND	Motor neurone disease
NAD	Nicotinamide adenine dinucleotide
NGS	Normal goat serum
NRS	Normal rabbit serum
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PGI_2	Prostacyclin
PM	Platelet membrane
PMSF	Phenylmethylsulfonyl fluoride
PPP	Platelet poor plasma
PRP	Platelet rich plasma
RABMFGM	Rabbit anti-(BMFGM)

(continued)

RABSA	Rabbit anti-(BSA)
RACS	Rabbit anti-(citrate synthase)
RAID	Rabbit anti-(isocitrate dehydrogenase)
RAPM	Rabbit anti-(platelet membrane)
RAXO	Rabbit anti-(xanthine oxidase)
RDWBM	Reconstituted dried whole bovine milk
SDS	Sodium dodecylsulphate
SMC	Smooth muscle cell
TCA	Trichloroacetic acid
TMB	3,3',5,5'- Tetramethylbenzidine
Tris	Trishydroxymethylamine
TXA_2	Thromboxane A_2
VLDL	Very low density lipoprotein
XO/D	Xanthine oxidase/deydrogenase

CONTENTS.

INTRODUCTION

1.0 HEART DISEASE

1.1 Introduction.

One hundred years ago Coronary Heart Disease (CHD) was virtually unknown. By 1925 it was still being discussed, in the Lancet, as an obscure medical curiosity. Now it kills more people in Britain than cancer, or any other known cause of death.

At the start of the eighties, CHD appeared destined to become the country's disease of the decade. Comparisons between nations (Vemura and Pisa, 1985) revealed that the countries of the United Kingdom were becoming increasingly isolated at the top of the International League table for CHD mortality (see Table 1, p2). Measures proposed to establish risk factors for the disease became a topic of concern. Epidemiological surveys (Dawber, 1980;Keys, 1980;Shaper et al, 1981) established blood pressure, serum cholesterol, relative body weight, and cigarette smoking as major risk factors. Other investigators (Hopkins and Williams, 1981) have added to this list which now contains over 200 entries.

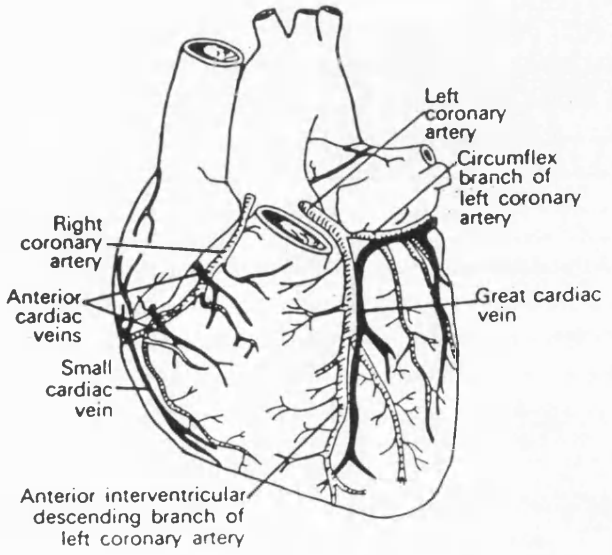
The heart is essentially a pump which circulates blood (about 7,000L/day) around the body. It does so by means of electrically stimulated contractions of its muscle, the myocardium, which receives its own blood supply from the coronary arteries (see Fig 1, p3).

Table1 Age-standardised mortality from ischaemic heart disease in 1980. Rates per 100,000 population aged 40-69 years.

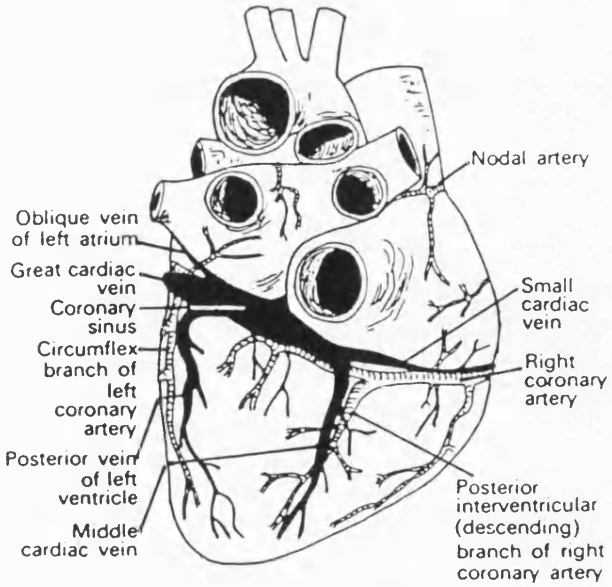
Males		Females	
Northern Ireland	630	Scotland	208
Finland	599	Northern Ireland	191
Scotland	592	New Zealand	178
Eire	499	Eire	160
England and Wales	482	England and Wales	136
New Zealand	468	Hungary	134
Czechoslovakia	438	Australia	133
Australia	421	Czechoslovakia	132
Hungary	410	U.S.A.	130
U.S.A.	398	Israel	128
Denmark	392	Finland	121
Canada	390	Canada	118
Norway	390	Denmark	112
Sweden	386	Bulgaria	100
Netherlands	323	Romania	93
West Germany	314	Sweden	90
Israel	314	Norway	86
Austria	293	Austria	80
Poland	282	Netherlands	78
Bulgaria	268	West Germany	75
Belgium	264	Belgium	72
Switzerland	219	Yugoslavia	71
Italy	212	Poland	66
Romania	202	Italy	53
Yugoslavia	197	Switzerland	47
France	137	France	30
Japan	65	Japan	24

Vemura and Pisa (1985)

Fig 1 The main arteries and veins of the coronary circulation.



Front or sternocostal view.



Back or diaphragmatic view.

From Bowman and Rand (1980), p22.4

In CHD, interruptions to the bloodflow result from a number of processes, including those which lead to a thickening of the artery walls, causing a narrowing of the arteries themselves. This process is known as atherosclerosis (see sect 1.2, p9). Uncertainty exists about the precise relationship between most CHD associated factors and the cellular development of atherosclerosis (Ross, 1986). Nevertheless it is indisputable that atherosclerosis can lead to severe occlusion of the coronary arteries (Gorlin et al,1986), giving rise to the following three principal manifestations of CHD;

1. Angina Pectoris, a gripping pain experienced in the chest, often extending to the neck and left arm under physiological exertion or emotional stress. The pain is the result of an inadequate supply of oxygen reaching the myocardium when contraction is required.

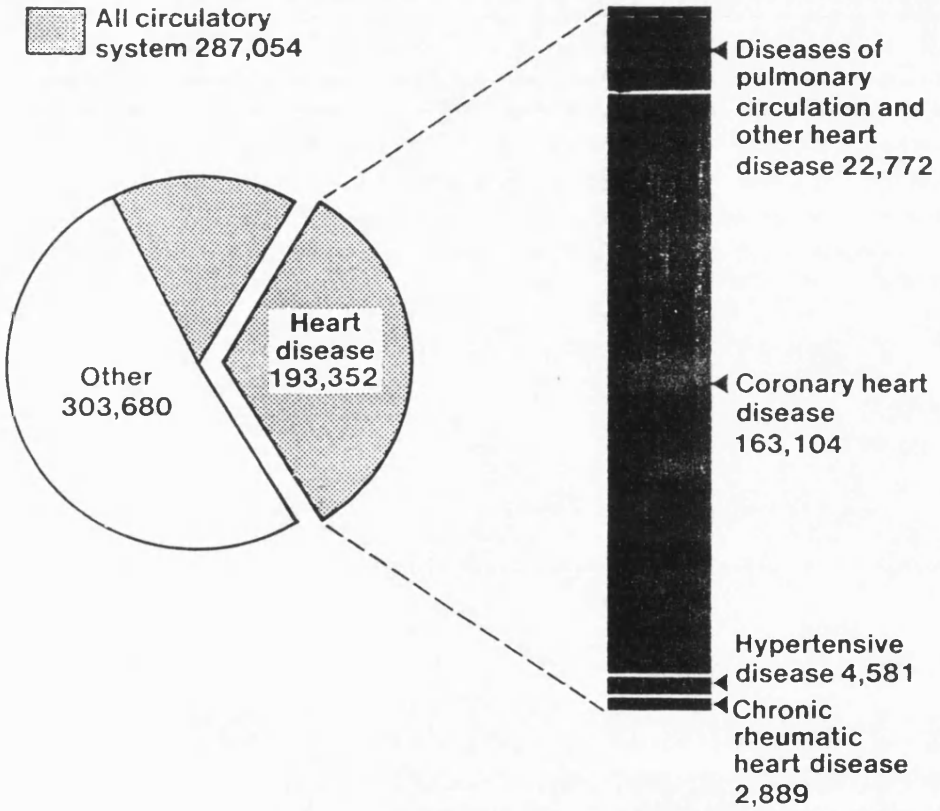
2. Myocardial Infarction (MI). Normal contraction of the myocardium cannot occur because of sustained loss of blood supply. The lack of oxygen in a particular area results in muscle necrosis, the extent of which is thought to be an important factor in influencing survival.

3. Sudden Cardiac Death. Here, a patient, often in good health, collapses entirely without warning (Eisenberg et al, 1986). Death is usually the result of ventricular fibrillation leading to pump failure (Mitchell, 1978).

Fig 2, p6, illustrates the large contribution (49%) that diseases of the circulatory system made to mortality in England and Wales in 1985. Conditions relating to the heart account for 67% of deaths within this group, the most significant disorder being CHD. Consequently, CHD caused 28% of the total mortality for England and Wales that year. Table 2, p7, shows the age distribution of mortality from CHD. The majority (80%) of deaths from this occur in persons aged 65 or over. Nevertheless, the disease remains a significant cause of premature mortality. Overall, it accounts for one third of male deaths under 65, but reaching two fifths between the ages of 45 and 65 (see Fig 3,p8).

CHD during the middle decades of life is far more common in men than in women (Stamler,1963;Barrett-Connor and Khaw,1988). With increasing age, this sex differential disappears (Fig 3, p8). One possible explanation is that increased endogenous oestrogen production may prevent development of the disease, and, after the onset of the menopause, this protection disappears (Oliver and Boyd,1959;Kennel et al,1976). However, the incidence of venous thrombosis in younger women is higher than in men from the same age group (O.P.C.S.,1982) and angina is more frequently experienced than in men (R.C.G.P.,1986). Bradley et al (1978) have reported that HDL levels increase with increasing doses of oestrogen and fall with increasing progesterone doses when studying females taking oral contraception (in general high HDL levels are

Fig 2 Causes of death in England and Wales for 1985.



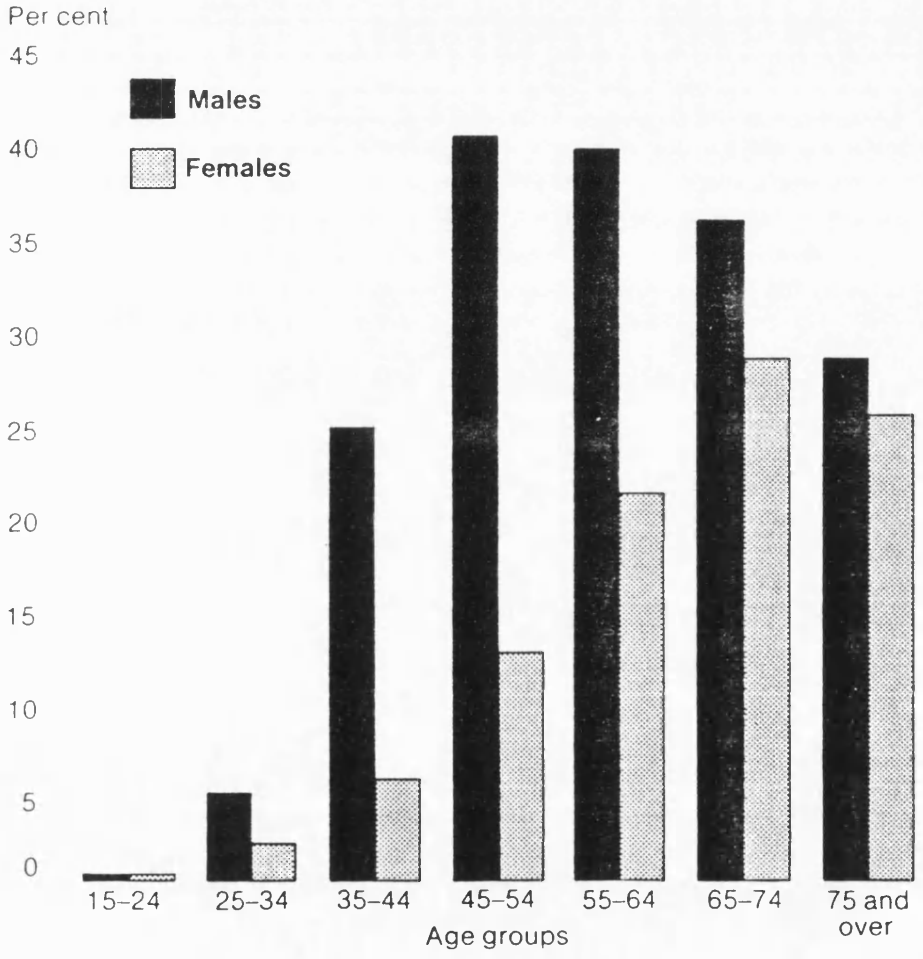
From Wells (1986)

Table 2 Age distribution of CHD mortality in England and Wales, 1985.

Age group	Males	Females	Total
Under 15	1	0	1
15-24	10	4	14
25-34	138	32	170
35-44	1,398	205	1,603
45-54	5,910	1,114	7,024
55-64	17,902	5,733	23,635
65-74	30,199	16,562	46,761
75+	36,068	47,828	83,896
All Ages	91,626	71,478	163,104

From Wells (1986).

Fig 3 Deaths from CHD as a percentage of all deaths at selected ages, England and Wales, 1985.



From Wells (1986).

considered to be protective against CHD (Gordon et al, 1989)).

1.2 Atherosclerosis

In 1904 Marchand recognised the consistent association of fatty degeneration and vessel stiffening. The term atherosclerosis was reintroduced (Athera = gruel, Sclera = hardening). Atherosclerosis is often associated with narrowing of the arteries, affecting large and some medium sized arteries, mainly the aorta, femoral, popliteal, renal, carotid, coronary, vertebral, and the circle of Willis (Bowman and Rand,1980). It is only the internal lining of the arteries which is susceptible to atherosclerotic lesions.

Atheroma will not cause any problems if the lumen allows sufficient blood to get through to the heart muscle. Several factors are now thought to lead to accelerated occlusion. For example, local bends in arteries can influence exactly where plaques develop (Mitchell and Schwartz,1965), and real problems of MI develop when the coronary artery suddenly becomes blocked because of any combination of the following:-

1. Rupture of an atheromatous plaque.
2. Thrombus formation.
3. Coronary artery spasm.

The walls of arteries contain three layers (see Fig 4, p11):-

A.The Intima consists of a single layer of flattened endothelial cells lining the lumen, and mounted on a basement membrane. Beneath this is loose fibrous elastic connective tissue and a well developed internal elastic membrane.

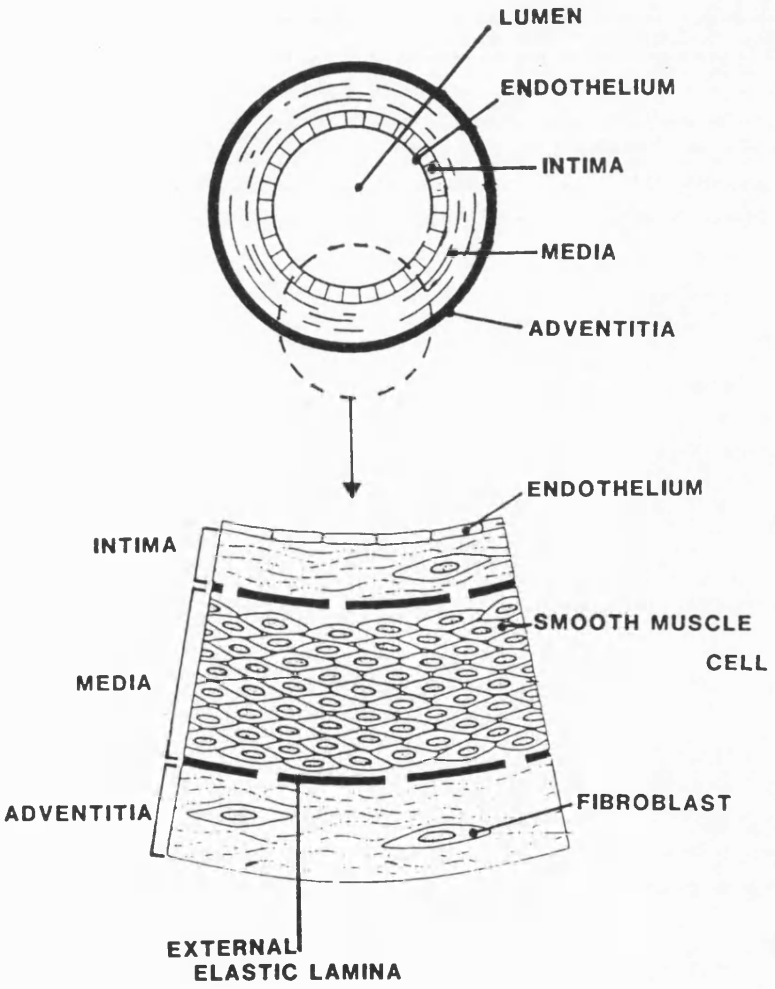
B.The Media comprises circularly arranged smooth muscle cells, together with elastin fibres in all but the smallest arteries.

C.The Adventitia contains a fibro-elastic tissue with a nerve plexus under sympathetic stimulation. Fibroblasts are present in this layer also.

The lesions of atherosclerosis can take two major forms, the fatty streak and the advanced lesion or fibrous plaque. The fatty streak can occur throughout life from as early as infancy, and is a flat lesion. Cholesterol esters are present in appreciable amounts, intracellularly, in both smooth muscle cells and macrophages, which are thereby transformed into so called foam cells. The advanced lesion or fibrous plaque (see Fig 5,p12) is found in young individuals with genetic forms of hyperlipidaemia, but in the apparently healthy population is found to increase in frequency with increase in age (Woolf,1982).

The fibrous plaque has four dominant pathological characteristics, Fig 6,p 12:-

Fig 4 Transverse section through a normal artery, expanded to show in more detail to show the composition of its layer structure.



Adapted from Taussig (1979) and Wells (1982).

Fig 5 Transverse section through a diseased artery, partially occluded by a fibrous plaque.

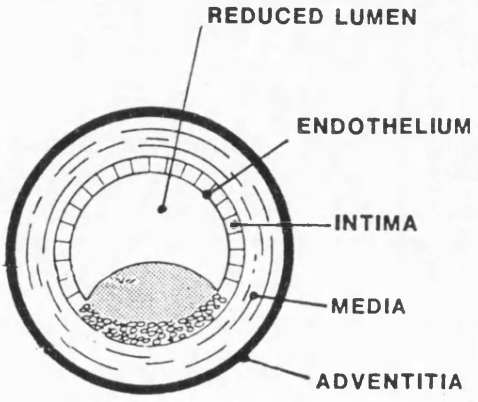
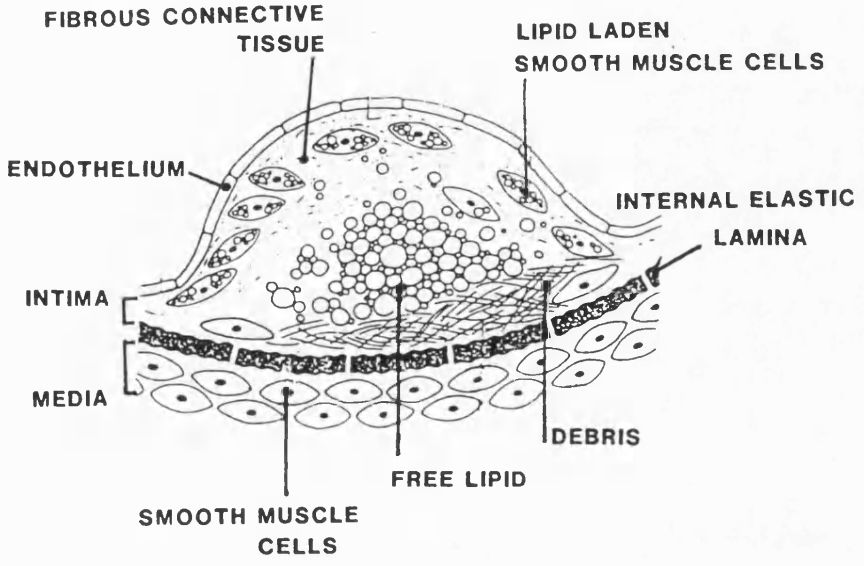


Fig 6 The pathological characteristics of the fibrous plaque.



Adapted from Taussig (1979) and Wells (1982).

1.Proliferation of smooth muscle cells, with variable, but large numbers of blood-derived macrophages, platelets, neutrophils, and lymphocytes.
2.The presence of the connective tissue matrix proteins and proteoglycans produced by smooth muscle cells.
3.Lipid accumulation in the form of foam cells within smooth muscle cells and macrophages.
4.Focal distributions of the lesions.

Later events in the history of the plaque may involve death of intimal smooth muscle cells and softening of the plaque base, possibly leading to ulceration, calcification, and lastly mural, or occlusive thrombosis (Forrester et al, 1987). Controversy exists concerning the relationship of the fatty streak and the fibrous plaque (Woolf,1982; McGill,1984). In recent years, another lesion, the gelatinous lesion, has also been implicated in the development of plaques (Haust,1971). It is generally considered to be a form of focal, intimal oedema.

1.3 The pathogenesis of atherosclerosis.

The processes involved in atherogenesis are still the subject of much debate. Table 3, p14-15 summarises some of the findings which have contributed to our current perception of the disease. According to our present understanding, atherosclerosis can be discussed under two headings; Hyperlipidaemia and thrombosis.

Table 3 Some major contributions of research into atherosclerosis.

AUTHOR	OBSERVATION
Crell, 1740	Basal pool in atherosclerotic plaque is akin to pus and represents the end point of an inflammatory process.
Von Haller,1755	Similar to that of Crell and re-introduced the term Atheroma.
Jenner, 1778	Correlation of anginal symptoms and artery disease.
Baillie, 1793; and Hodgson, 1815	Association between lesions in coronary arteries and at least one of the symptoms of myocardial ischaemia.
Von Rokitansky,1844	Plaques contain deposition of blood constituents, mainly fibrin, on the luminal surface of the arterial wall.
Vogel,1847	Plaques contain large amounts of cholesterol.
Virchow,1856	Lesion is subendothelial and arises from the loosening of the connective tissue ground substance of the lumen as a result of "imbibition" of constituents of passing blood.
Thoma,1883	Primary fault is a localised weakness in the media and the consequent aneurysmal dilation evokes a secondary connective tissue proliferation.
Anitschkow and Chalatov,1913	Fatty lesions in rabbits can be produced on provision of a diet rich in egg yolk.
Duguid,1946	Mural thrombi are organised in atherosclerotic plaques.

(continued)

Crawford and Levene,1953	Medial thinning is an accompaniament of the atherosclerotic plaque.
Poole *et al*, 1958	Removal of the endothelium gives rise to intimal lesions and smooth muscle cell proliferation.
Carstairs,1965	Identification of platelets in atherosclerotic plaques.
Benditt and Benditt,1973	Smooth muscle cell proliferation within fibrous plaques is monoclonal.
Ross *et al*, 1974	Platelet-Derived growth factor causes smooth muscle cell proliferation.
Brown *et al*, 1975	Free and esterified cholesterol is regulated by an LDL receptor.
Mustard and Packham,1975	Mural thrombi contain smooth muscle cells and lipids.
Wolinsky *et al*, 1975	Lysosomes in atherosclerotic plaques have increased lipid content.
Schwartz and Benditt,1976	Increase in cellular turnover in endothelial foci gives rise to an increase in trans-edothelial permeability.
Monacada *et al*, 1976	Arterial walls are protected against platelet deposition by prostacyclin.

The involvement of hyperlipidaemia in atherosclerosis can be inferred from the following:-

(a) Atherosclerotic lesions contain more cholesterol than surrounding areas

(b) Genetic conditions, causing elevated lipid levels in serum, predispose certain individuals to premature heart disease (Brown and Goldstein,1984).

(c) Diets, high in cholesterol and saturated fats, are associated with populations in which heart disease is prevalent (Keys,1970;Shekelle et al,1981), and reducing plasma cholesterol levels in hypercholesterolaemic men, reduces their risk of having an MI (L.R.C.P.,1984;Vines,1989).

Involvement of thrombosis in atherosclerosis is suggested by the following observations:-

(a) Atherosclerotic plaques are associated with mural thrombi containing varying amounts of platelets and fibrin.

(b) Changes associated with early atherosclerotic lesions are influenced by platelets (Duguid,1948; Moore et al,1976).

1.3.1 Lipids and atherosclerosis.

Lipids and cholesterol are carried in the bloodstream as complex lipoproteins, named according to their densities and with the compositions shown in Table 4, p 17. Various proteinaceous components of lipoproteins,

Table 4 Composition of the plasma lipoproteins

Type	Density (g/cm^3)	Major Polypeptides
Chylomicrons	<0.94	apo A,B,C
Very Low Density Lipoproteins (VLDL)	0.94 - 1.006	apo B,C,E
Low Density Lipoproteins (LDL)	1.006 - 1.063	apo B
High Density Lipoproteins (HDL)	1.063 - 1.210	apo A

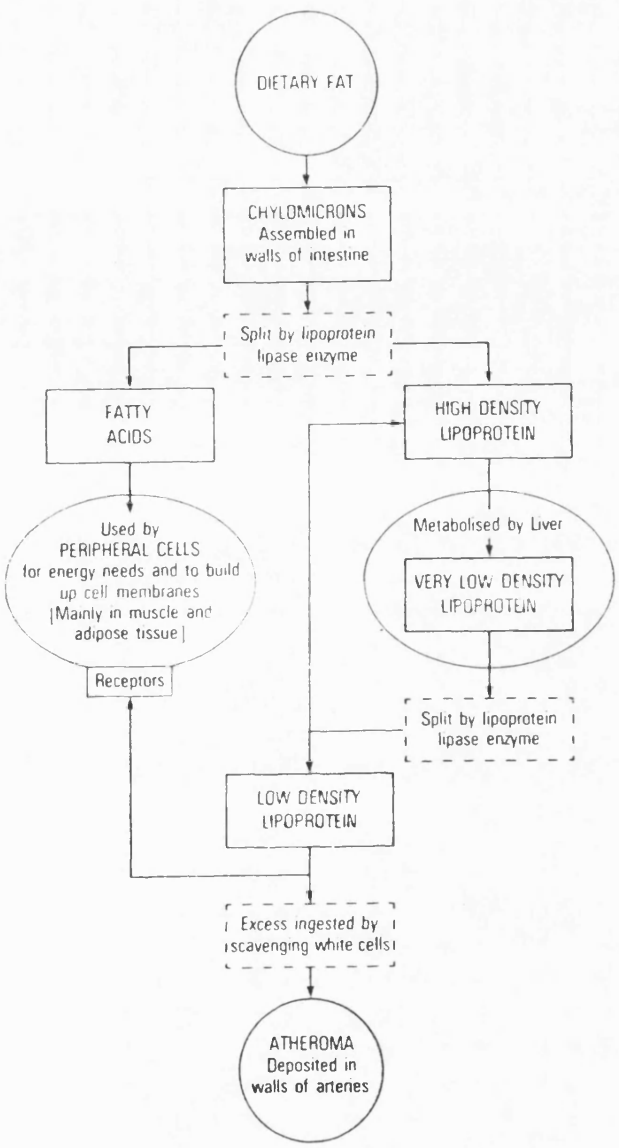
From Stryer (1981), p470

lipoproteins, termed apoproteins, are designated A-E. The two lipoproteins most relevant to heart disease are Low Density Lipoprotein (LDL) and High Density Lipoprotein (HDL). The trafficking of lipoproteins in the body is explained in Fig 7, p19-20. Briefly, the role of LDL is to transport cholesterol to peripheral tissues and regulate de novo cholesterol synthesis at these sites. One role of HDL is to transport cholesterol from peripheral tissues to the liver. Non-hepatic cells have specific LDL receptors on their surface. Following binding to the cells LDL is endocytosed and metabolised. Much of our current understanding of this phenomenon results from the work of Brown and Goldstein (1986), who have speculated that, if LDL receptors in arterial walls are saturated with LDL (i.e. there is an excess in the blood) then both cholesterol and its esters may accumulate in arterial walls by non-physiological bulk transfer systems. It is possible that an increase in the number of cholesterol molecules of the endothelial plasma membrane may affect its physical properties (Jackson and Gotto,1976). Evidence for the hypothesis stems from the genetic disorder, Familial Hypercholesterolaemia, in which there is a defect or absence of the LDL receptor. In addition, people suffering from this condition are prone to heart attacks from as early an age as two. Diet induced hypercholesteroleamia also leads to the characteristic lesions of the fatty streak (Gerrity,1981;Faggiotto et al,1984).

Explanation of Fig 7

Dietary fat and cholesterol are absorbed through the wall of the gut and assembled there as chylomicra. Lipoprotein lipase, present on the capillary walls of fatty and muscle tissue, is activated by the apoprotein of the chylomicron and the free fatty acids and monoglycerides of the chylomicron, that are released by this action, are used by adjacent cells. The remaining components of the chylomicron form HDL, which in turn circulates in the blood and is captured by receptors in the liver. Cholesterol arriving at the liver in this way, or metabolised by the liver itself, is packaged into VLDL which, after circulation, is acted upon by another lipoprotein lipase to form HDL and LDL.

Fig 7 Lipid and cholesterol transport in the human body.



From Heller (1987)

The relationship of plasma concentrations of HDL and LDL to vessel wall disease has been studied by many workers. Coronary artery angiography has suggested that the severity of coronary artery disease increases with increasing LDL concentrations, and decreases with increasing concentrations of HDL (Jenkins et al, 1978). In vitro work has suggested a pathological mechanism for this (Bondjers and Bjorkerud,1973). Atherosclerotic regions of rabbit aorta, showed an increase in cholesterol when incubated in media containing LDL. In contrast, HDL promoted a slight decrease of intracellular cholesterol. Heparin, exposed on disruption of endothelia can also bind LDL by means of its apoprotein (B and E) components (Weisgraber et al,1986). It is also apparent that many LDL forms exist among individuals and some may be more atherogenic than others (Sniderman et al,1980; Rudel et al,1985). Recently Cathcart et al, (1985) have observed that LDL exposed to macrophages is oxidised and is toxic to fibroblasts in culture, if oxidation occurs in vivo, it may be an important source of endothelial assault. It has been proposed (Wolinsky et al,1975) that a decrease in the activity of smooth muscle cell lysosomes, particularly the enzyme cholesterol ester hydrolase, may cause cholesterol accumulation. The cell may then die with the release of undegraded cholesterol into its environment.

1.3.2 Platelets, thrombosis and atherosclerosis .

Platelets were described more than 100 years ago (Bizzozero,1882). In their normal state, they are convex discs, heterogeneous in size, but with an average diameter of 2.5μ (Thompson et al,1982). The larger platelets are those that have been newly formed and, are most active. Platelets are derived from large precursor sten cells, megakaryocytes (Levine,1982), under the influence of the hormone thrombopoietin (Abildgard and Simone,1967; Cooper,1970). Megakaryocytes are found in the lung and bone marrow and it has been calculated that one megakaryocyte is capable of releasing between 2,000 and 7,000 platelets in its lifetime (Thiery and Bessis,1956; Cronkite,1958).

Fig 8, p23-24, illustrates the ultrastructure of platelets. Despite the absence of a nucleus, platelets possess many of the features of classic inflammatory cells (Page,1988). They are capable of phagocytosis and chemotaxis, and can release a variety of mediators that augment inflammatory recruitment (Nachman and Weksler,1980). A defence role in killing parasites has also been proposed (Copley,1979). However, their contributions to the above processes have been overshadowed by their involvement in Haemostasis.

Wintrobe (1974) describes how vascular injury results in adherance of platelets to the vessel wall. Activation occurs, and platelets change shape, spread, secrete the contents of their storage granules and aggregate. The

Key to Fig 8

E.C. - Exterior coat or glycocalyx

C.M. - Unit membrane

S.M.F - Microfilaments

O.C.S - Open cannalicular system

M.T. - Microtubule bundles

Gly - Glycogen granules

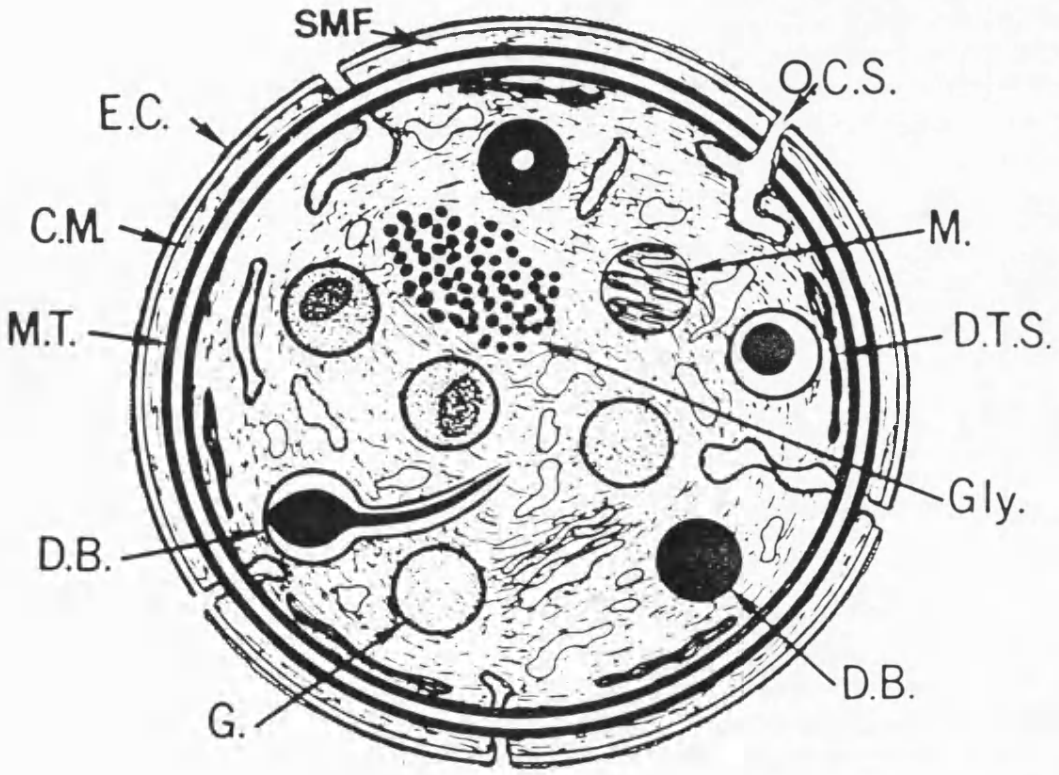
M. - Mitochondria

G. - α-granules, lysosome-like organelles containing platelet factor 4, and PDGF

D.B. - Dense bodies, containing ADP, ATP, Calcium, Serotonin, Pyrophosphate, and Antiplasmin

D.T.S - Dense-Tubule system akin to the Endoplasmic or Sarcoplasmic reticulum

Fig 8 The ultrastructure of the human platelet.

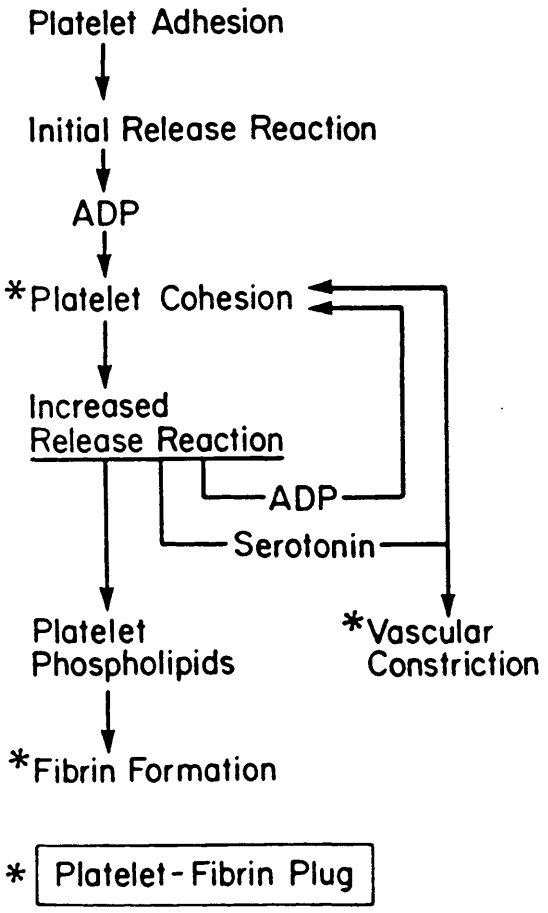


From Johnson (1971), p48.

adhesion of circulating platelets to subendothelial fibres is mediated by a receptor on the platelet surface, Glycoprotein Ib (GpIb), which binds to collagen (George et al,1984) and Von Willebrand factor (VWF) (Jaffe et al,1973; Weiss et al,1973). Adherent platelets release ADP, from secretory granules, which promotes platelet aggregation. Aggregation reveals a phospholipid factor which is required for the conversion of prothrombin to thrombin, which further stimulates platelets, by hydrolysis of its own receptor Glycoprotein V, on the platelet surface (Philips and Agin,1977). Thrombin helps to stabilise the platelets in a haemostatic plug. In addition platelets release the protein, Platelet Factor 4, which promotes clotting by neutralising the action of Heparin. Fig 9, p 26, summarises the contribution of platelets to the blood clotting sequence.

In the normal response to endothelial injury, platelets quickly adhere to the damaged area, as already described. The healthy vessel wall is believed to be protected from such adhesion by the powerful antiaggregator and vasodilator prostacyclin (PGI2). Thromboxane (TXA2), in contrast, is an aggregatory substance, derived from platelet endoperoxides (Raz et al, 1977), and it is thought that there exists a homeostatic balance between PGI_2 and TXA_2 in vivo. Interestingly, it has been reported (D'Angelo et al,1978) that human atherosclerotic plaques do not

Fig 9 The involvement of platelets in the formation of a fibrin plug.



From Murano and Bick, 1980.

generate PGI_2. Fig 10, p28, illustrates the pathways of PGI_2 and TXA_2 production.

In atheroma, platelet deposition apparently fails to self-limit. Platelets and lipids infiltrate the inner layer of the vessel wall. LDL is known to bind to glcosylaminoglycans, particularly dermatan sulphate (Iverius, 1973). Smooth muscle cells (SMC), are seen to migrate from the media; mainly in response to PDGF, released by activated platelets. Although the process may stop at this stage and new endothelium can grow over any deposits, another plaque will often form over the first one. The process is extremely complicated, probably involving also similar growth factors, released by monocytes (Shimokado et al,1985), endothelial cells (Leibovich and Ross,1976; Gajdusek et al,1980), and smooth muscle cells themselves (Seifert et al,1984), see Fig 11, p29. Moreover, the endothelium can release heparan sulphate, a potent inhibitor of PDGF function (Rosenberg et al,1985). Clearly, we are dealing with a complex homeostatic mechanism.

The "Response to Injury" hypothesis of atherosclerosis.

The response to injury hypothesis proposed by Ross suggests that injury to the endothelium is the initiating event in atherogenesis and at least two different mechanisms may be in operation.

The first includes the hypercholesterolaemic and thrombotic elements already mentioned. Hyper-

Fig 10 Major prostaglandin synthesis in platelets and normal endothelium.

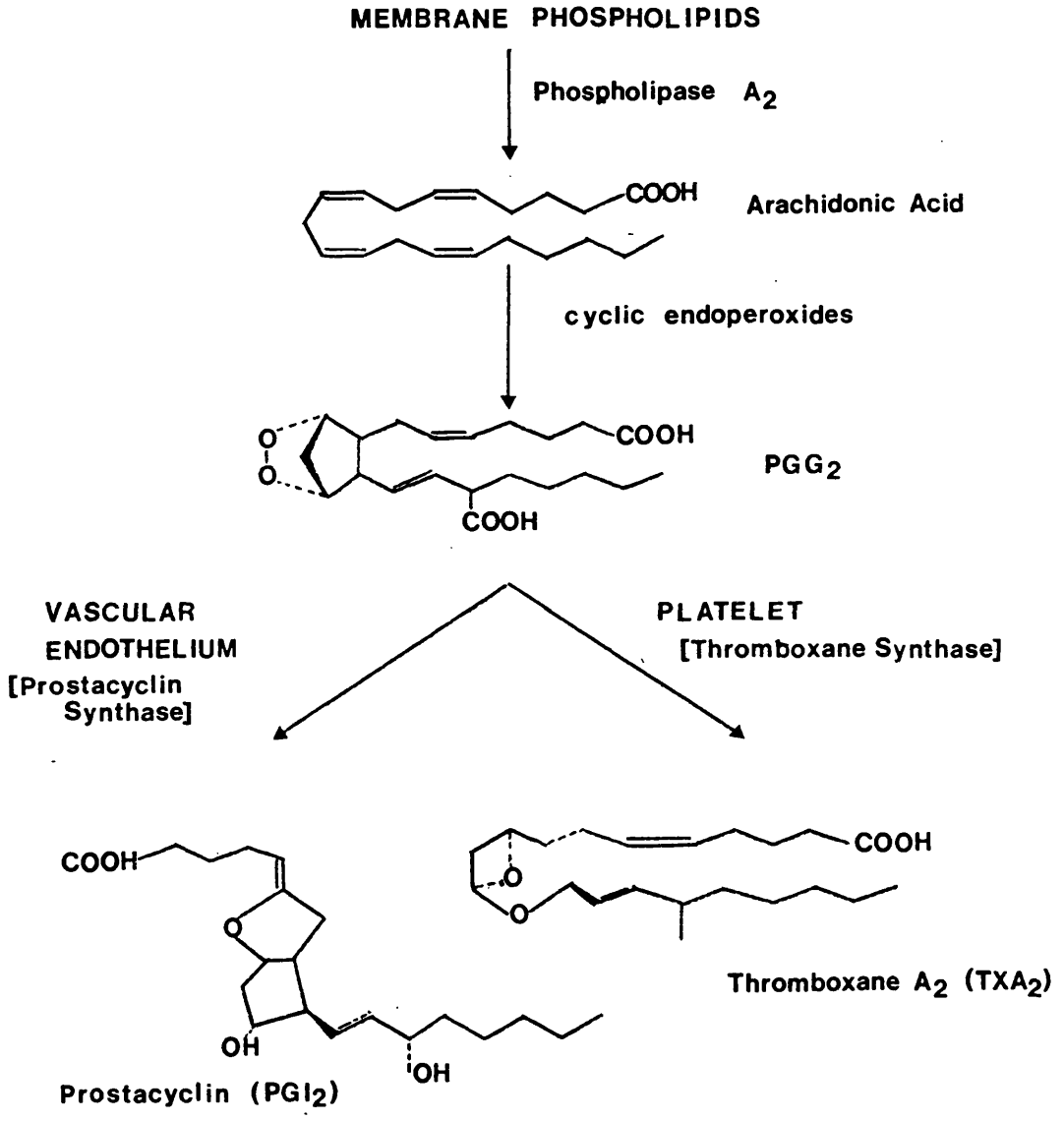
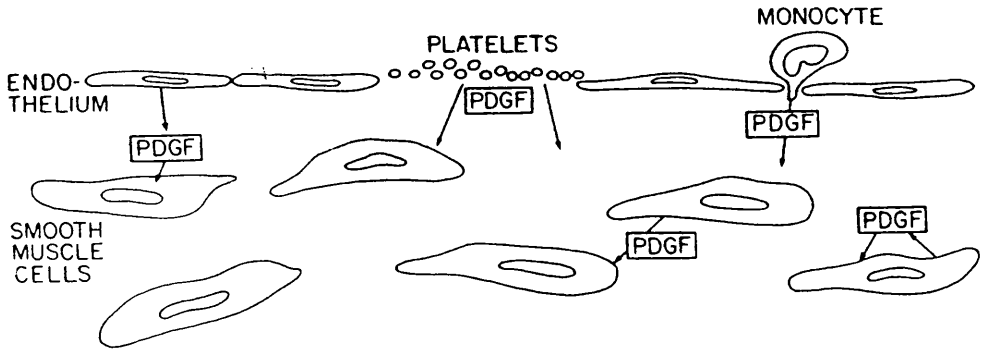


Fig 11 Possible pathways for stimulation of vascular smooth muscle cells by PDGF.



1. Endothelial loss promotes platelet adherance with consequent release of PDGF.

2. Endothelial cells produce a PDGF mitogen which <u>in vitro</u> stimulates smooth muscle cells, and release a factor which inhibits PDGF action.

3. Intimal smooth muscle cell production of PDGF.

4. Blood monocyte production of PDGF

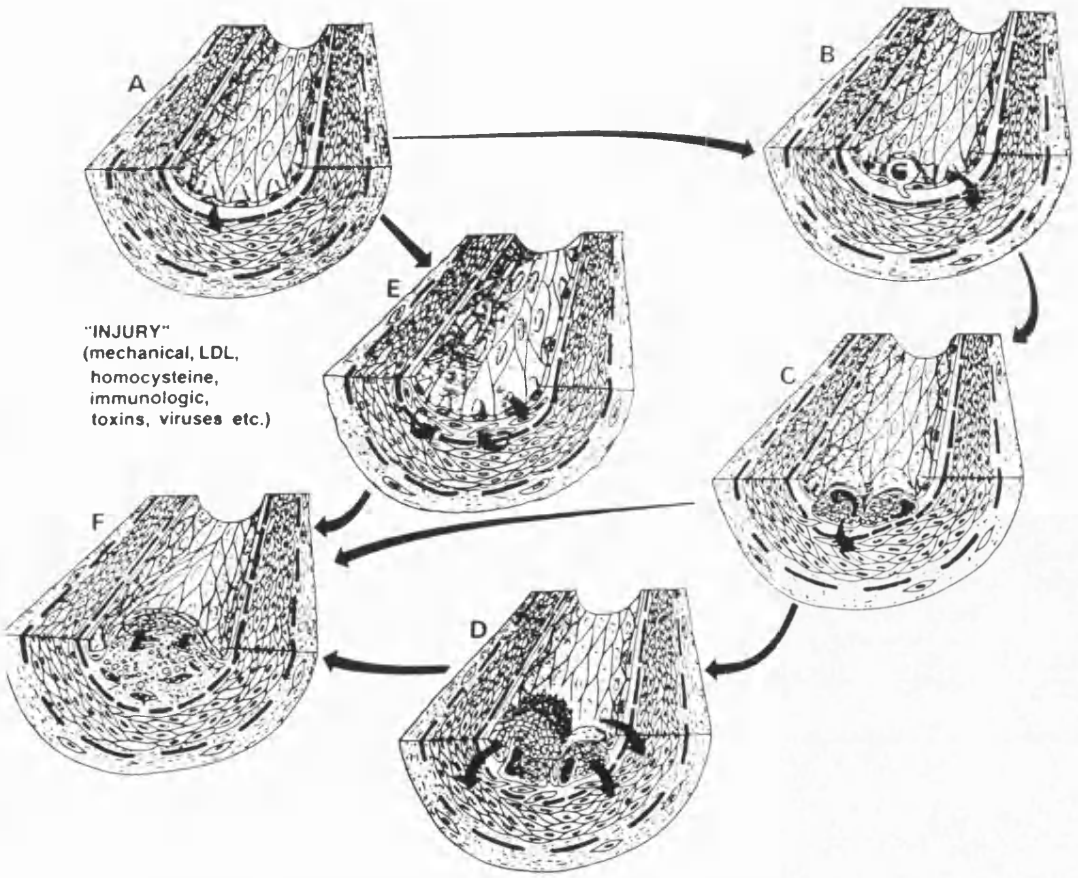
Reidy (1986), p115.

cholesterolaemia may lead to changes in the endothelium, as already stated, involving detachment of individual endothelial cells themselves, replacement occurring by spreading of adjacent cells (Reidy and Schwartz,1984), or by an increased turnover of adjacent cells (Bjorkerud and Bondjers,1972). This may result in monocyte adherance and subendothelial migration, accumulation of lipid to form foam cells and gradual accumulation of SMC to form fatty streaks (Faggiotto et al,1984). At some sites endothelial cells may separate, exposing foam cells and connective tissue to which platelets may bind and initiate SMC migration (Faggiotto and Ross,1984), by the release of PDGF. However areas of denuded endothelium seem to be a rare occurrance in man (Bylock et al,1979; Reidy,1985). It has therefore been questioned whether endothelial denudation plays a significant role in the initiation of the atherosclerotic process, but may be involved in its progression.

The second pathway involves direct stimulation of endothelium, which may release growth factors, vasoactive agents, or growth inhibitors, possibly inducing other cellular activities such as smooth muscle migration (Ross,1986).

The processes described above are summarised in fig 12, p 31.

Fig 12 Possible mechanisms in the development of atherosclerosis.



The responses to injury hypothesis. Advanced intimal proliferative lesions of atherosclerosis may occur by at least two pathways. The pathway demonstrated by the clockwise (long) arrows to the right has been observed in experimentally induced hypercholesterolemia. Injury to the endothelium (*A*) may induce growth factor secretion (short arrow). Monocytes attach to endothelium (*B*), which may continue to secrete growth factors (short arrow). Subendothelial migration of monocytes (*C*) may lead to fatty streak formation and release of growth factors such as PDGF (short arrow). Fatty streaks may become directly converted to fibrous plaque (long arrow from *C* to *F*) through release of growth factors from macrophages or endothelial cells or both. Macrophages may also stimulate or injure the overlying endothelium. In some cases, macrophages may lose their endothelial cover and platelet attachment may occur (*D*), providing three possible sources of growth factors—platelets, macrophages, and endothelium (short arrows). Some of the smooth muscle cells in the proliferative lesion itself (*F*) may form and secrete growth factors such as PDGF (short arrows).

An alternative pathway for development of advanced lesions of atherosclerosis is shown by the arrows from *A* to *E* to *F*. In this case, the endothelium may be injured but remain intact. Increased endothelial turnover may result in growth factor formation by endothelial cells (*A*). This may stimulate migration of smooth muscle cells from the media into the intima, accompanied by endogenous production of PDGF by smooth muscle as well as growth factor secretion from the "injured" endothelial cells (*E*). These interactions could then lead to fibrous plaque formation and further lesion progression (*F*).

From Ross (1986) p496.

1.3.3 Immunological aspects of atherosclerosis

In addition to the Ross hypothesis, the importance of the endothelium in the development of atherosclerosis was first suggested in animal experiments by Poole et al (1958), Baumgartner (1963) and Bjorkerud and Bondjers (1971). Removal of the endothelium led to development of intimal lesions resembling atherosclerotic lesions. These were characterised by SMC proliferation, connective tissue formation and lipid deposition. Experimentally it is possible to cause endothelial damage in a variety of ways, including immunologically (Levy,1967; Minick and Murphy,1973). Rabbits receiving a cholesterol diet, and injected with a foreign protein, which elicited immune complex formation, showed a rapid increase in arterial lesions. The resulting enhancement of lipid accumulations, within the arterial intima, was greater than with either insult alone (Levy,1967). In the absence of cholesterol, however, immune complexes (CICs), would only produce lesions if vasoactive amines, complement and leucocytes were present (Minick and Murphy,1973).

Any soluble or insoluble antigenic material can form CICs if it is released in sufficient quantities at a time when specific antibodies are present in the plasma (Fig 77,p249). Generally, it is the IgG, IgA, and IgM classes which form complexes, very little is known about IgD, or IgE CICs. The nature and proportions of the antigen and antibody forming the complex determine its

fate in the body (van Es et al,1984). Larger complexes are cleared by macrophages, although precipitation may occur in the tissues. Very small complexes, on the other hand, pass harmlessly out through the urine. Intermediate sized complexes, found in moderate antigen excesss, tend to stick to walls of blood vessels. Their size is favoured by low antigen valency and low amounts of low affinity antibodies (Playfair,1982) and have a size of 1,000kD (Cochrane and Hawkins,1968). The latter group have also shown that CICs will only penetrate the endothelial lining of blood vessels if the vascular permeability is increased. The permeability of various blood vessels to CICs may vary with the haemodynamic forces within the blood vessel, and the structure of the internal endothelial lining of the blood vessels themselves. Post capillary venules are susceptible to CIC deposition, but it is less common in arterial walls where usually deposition is at sites of high shearing stress i.e. at arterial bends and bifurcations (Roitt,1988).

Immune complexes can activate complement and cause release of inflammatory mediators and chemoattractants. Histamine and bradykinin (Arfors et al,1979) induce contraction of endothelial cells, causing transient endothelial gaps to develop. Material exceeding 1,000kD can be trapped under the endothelium after the endothelial cells resume their original close apposition. These may attract Polymorphonuclear leucocytes (PMN), releasing destructive enzymes and oxy-

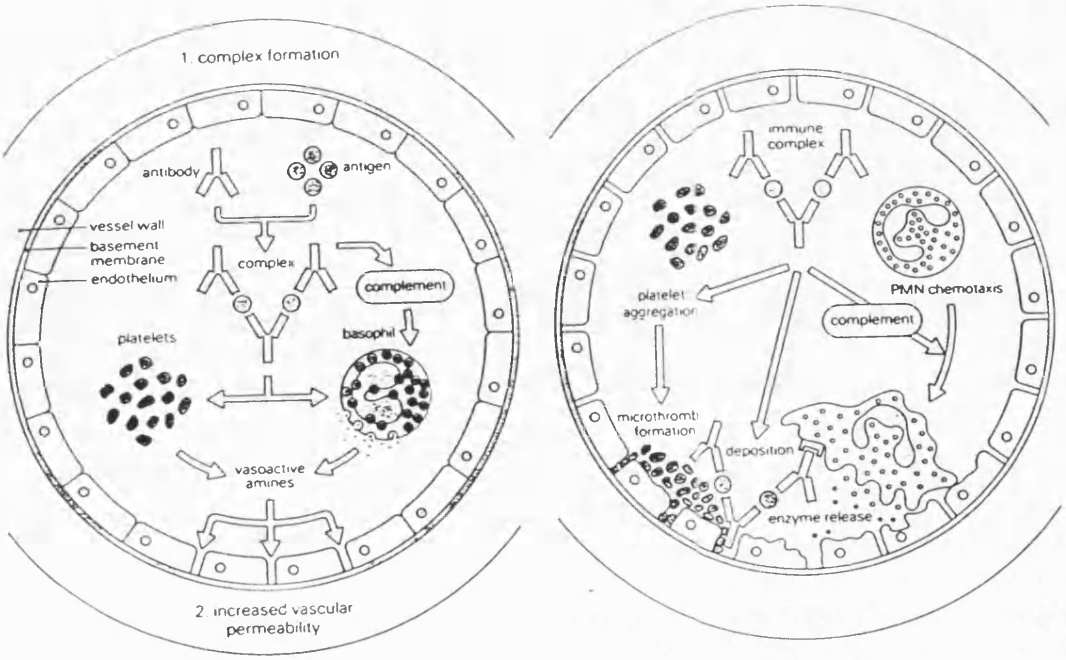
species, which may damage surrounding tissue. The extent of the damage and the resulting symtoms will depend upon various factors including whether or not exposure to the offending antigen is brief or repeated. Recently vimentin, a cytoskeletal component of endothelial cells has been shown to bind IgG by an Fc reaction (Hannson et al,1980,1984). Linder (1981) has pointed out the possibility of intracellular complement activation. Hannson et al, (1981) have also shown that monocyte adhesion to the vessel wall is reduced if IgG depleted serum is used in the media. Fig 13, p 36, illustrates some of the consequences of CIC deposition in arteries. Inflammatory changes occur co-existant with atherosclerotic lesions and inflammation or oedema of the vessel wall could provide an alternative mechanism for reduction in the arterial lumen (Shimamoto,1963; Shimamoto and Numano,1969). Virchow (1856) first suggested that atherosclerotic lesions were attributable to chronic irritation, and intimal oedema was observed by him too. Chronic inflammatory microlesions have also been documented in sudden unexpected heart attacks (Haerem,1975). Aortic and coronary arteritis are important consequences of serum sickness in man and animals (Saphir and Gore, 1950, Zak et al,1952). The depositon of CICs in the arterial wall may lead to inflammation which would increase endothelial permeability and possibly lead to lipid entry. Fig 13 also illustrates some of the immune reactions possibly involved in atherosclerotic injury. The work of Levy

(1967) showed that the atherosclerotic lesions of rabbits, pre-injected with foreign protein, were similar to that found in man. But, in humans, the most obvious association between immunologic injury and atherosclerosis is found in coronary artery lesions that can occur in donor hearts after cardiac allo-transplantation (Thompson,1969; Clark et al, 1973).

Immune complexes may form from a variety of antigens which may include infecting micro-organisms (Cunningham and Pasternak,1988), vaccines, antibiotics, drugs, foodstuffs (Minick,1976),tobacco (Harkavy and Perlman, 1964) and lipoproteins (Beaumont and Beaumont, 1978). A repeated or prolonged injury by formation of complexes from each of the above could illicit the local reactive changes mentioned above.

The involvement in atherosclerosis of pathogenic immune complexes, formed from dietary antibodies (particularly anti-milk antibodies) was proposed by Davies et al in 1969, he showed that anti cows' milk antibodies were increased in patients with CHD and postulated that this was a consequence of an abnormal immune state, akin to immune complex disease (Davies,1969; Poston and Davies,1974).

Fig **13** The effects of immune complex deposition in blood vessel walls.



Deposition of immune complexes in blood vessel walls I. Antibody and antigen combine to form immune complexes (1). The complexes act on complement (to release C3a and C5a), which in turn acts on basophils to release vasoactive amines. The complexes also act directly on basophils and platelets (in humans) to produce amine release. The amines released include histamine and 5–hydroxytryptamine, which cause endothelial cell retraction and thus increased vascular permeability (2).

Deposition of immune complexes in blood vessel walls II. With increased vascular permeability, complexes become deposited in the vessel wall. The complexes induce platelet aggregation and complement activation. The platelets aggregate to form microthrombi on the exposed collagen of the basement membrane of the endothelium. Polymorphs (PMN) attracted to the site by chemotactic complement peptides cannot phagocytose the complexes and so release their lysosomal enzymes to the exterior of the cell causing damage to the vessel wall.

From Roitt et al, (1985), p21.3.

1.4 DAVIES HYPOTHESIS

Initial studies by Davies (1958), at Carmarthen, Wales, investigated the mobility of erythrocytes by using an Abrahamson electrophoresis cell. He concluded that electrophoretic mobility decreased with increasing age of the donor. However, when erythrocytes were washed in saline, this age-dependent phenomenon disappeared, and, moreover, it subsequently transpired that mobility was a function of atheroma-related disease rather than of age.

An investigation followed, looking at 20 coronary occluded patients, and 20 age-matched control patients. A significantly raised erythrocyte migration time was found in the coronary-occluded patients (see Table 5, p 38, Davies,1958). Independent confirmation of this phenomenon followed (Begg et al,1966). Davies argued that the property of plasma that affects electrophoretic mobility of erythrocytes is likely to affect lipid particles in a similar fashion. This was reported to be the case (Davies,1959), see Table 6, p39, and it was shown that the electrophoretic migration of erythrocytes was related to the migration of chlyomicra. Davies also showed, not only that the erythrocyte migration time did not relate to plasma cholesterol levels (see Fig 14, p40), but also that such migration gave a much more satisfactory index of atheroma than did cholesterol levels (Davies and Clark,1961).

Further studies in 1967, showed a highly significant

Table 5 Comparison between erythrocyte migration times of coronary heart disease and control patients.

CORONARY PATIENTS

Pair No.	Age (yr.)	Condition	Mean erythrocyte migration times (sec.)	Standard deviation	Number of days observed
1	38	Coronary occlusion	81	25·8	6
2	42	,, ,,	79	18·3	6
3	50	,, ,,	105	44·0	9
4	50	,, ,,	96	26·0	5
5	51	,, ,,	95	15·7	5
6	52	,, ,,	102	24·0	10
7	52	,, ,,	77	21·0	4
8	52	,, ,,	86	14·3	5
9	53	,, ,,	78	16·6	6
10	54	,, ,,	87	17·0	5
11	54	,, ,,	91	12·3	5
12	55	,, ,,	100	8·7	5
13	55	,, ,,	84	24·3	6
14	56	,, ,,	79	14·0	5
15	59	,, ,,	93	12·0	5
16	60	,, ,,	74	15·4	6
17	61	,, ,,	93	13·6	5
18	64	,, ,,	91	14·6	5
19	67	,, ,,	77	20·4	6
20	67	,, ,,	70	17·5	6

Mean age = 54·6 yr..

CONTROL PATIENTS

Pair No.	Age (yr.)	Condition	Mean erythrocyte migration times (sec.)	Standard deviation	Number of days observed	Difference between means
1	38	Mitral stenosis	78	8·0	6	3
2	42	Normal	85	7·5	5	−6
3	51	Normal	80	18·3	6	25
4	53	Normal	64	7·0	5	32
5	56	Gastric ulcer	96	17·3	6	−1
6	51	Hypertension	67	16·0	6	35
7	52	Duodenal ulcer	69	19·3	6	8
8	48	Duodenal ulcer	76	13·1	5	10
9	53	Normal	73	10·8	5	5
10	57	Cholecystitis	56	5·1	5	31
11	58	Normal	99	24·2	4	−8
12	60	Gastric ulcer	77	10·5	5	23
13	55	Cor pulmonale	89	15·6	4	−5
14	57	Gastric ulcer	65	4·2	5	14
15	62	Chronic bronchitis	75	22·1	5	18
16	62	Pulmonary embolism	73	9·8	6	1
17	58	Hypertension	86	20·5	5	7
18	59	Pulmonary embolism	71	10·0	5	20
19	63	Normal	82	14·0	6	−5
20	72	Normal	63	3·6	4	7

Mean age = 55·3 yr.. Total = 214.

Mean difference in erythrocyte migration times between pairs = $\frac{214}{20}$ = 10·7

S.E. of mean = ± 3·01

∴ " t " = 3·55, degrees of freedom = 19

∴ 0·01 > p > 0·001

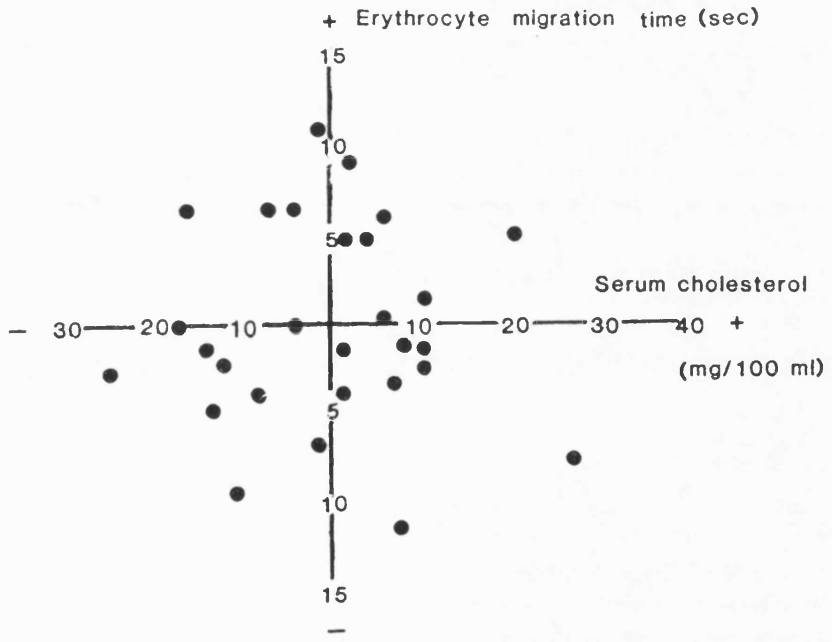
From Davies (1958).

Table 6 Comparison between chylomicron and erythrocyte migration times on samples obtained from 25 hospital in-patients (n = 3).

Patient Number	Chylomicron migration times (sec).		Erythrocyte migration times (sec).	
	Mean	Standard Error	Mean	Standard Error
1	52	1.1	49	3.1
2	53	3.3	56	2.4
3	55	3.7	52	2.7
4	57	3.6	55	1.6
5	58	2.4	60	3.5
6	60	4.7	54	4.2
7	63	3.6	65	3.8
8	64	5.0	56	2.9
9	66	4.5	61	2.2
10	73	2.0	61	4.9
11	75	6.1	68	3.2
12	77	4.5	74	3.7
13	79	1.5	65	2.7
14	80	3.9	79	3.9
15	82	2.8	70	5.1
16	84	5.1	81	2.7
17	85	2.6	70	4.2
18	92	4.1	85	2.9
19	93	3.3	83	3.2
20	97	3.1	85	2.5
21	100	4.3	97	5.2
22	101	6.8	89	2.4
23	102	5.7	82	4.6
24	102	7.1	89	4.1
25	103	2.5	97	4.6

From Davies (1959).

Fig 14 Variations in erythrocyte migration time and serum cholesterol about the mean level characteristic for each patient.



From Davies (1959).

correlation between erythrocyte migration times and platelet adhesiveness (Davies and Lloyd,1967), see Fig 15, and Table 7,p 42), a factor known then to be associated with CHD (Florey,1960;McDonald,1960).

The next series of investigations were designed to discover the nature of the plasma agents that were affecting the above factors in CHD patients. It was found that IgG was raised in a series of 50 patients, compared to 78 controls (Davies and Clark,1968) (see Fig 16, p43). Furthermore, of the heart diseased patients examined serially, the increase was not secondary to infarct itself i.e. antibodies were not being formed to neo-antigens. An independent group, Gray et al, (1978), also confirmed Davies' findings. The results of these findings suggested that the mechanism reducing the plasma surface activity was immunological in nature and that the tendency for circulating blood particles to adhere together and to blood vesssel walls could be the expression of immunoadherence.

Davies decided to look at possible exogenous antigens to which CHD patients were being sensitised and focussed on food antigens which were, by then, known to be intestinally absorbed in macromolecular form (Schloss,1924/25; Gunther et al,1960; Parish et al,1960; Taylor and Truelove,1961, see also Introduction, Sect.3, p60). He found that antibodies to cows' milk were elevated in CHD (Davies et al,1969). This was extremely interesting considering milk itself has long

Fig 15 The relationship between erythrocyte migration time and platelet adhesiveness.

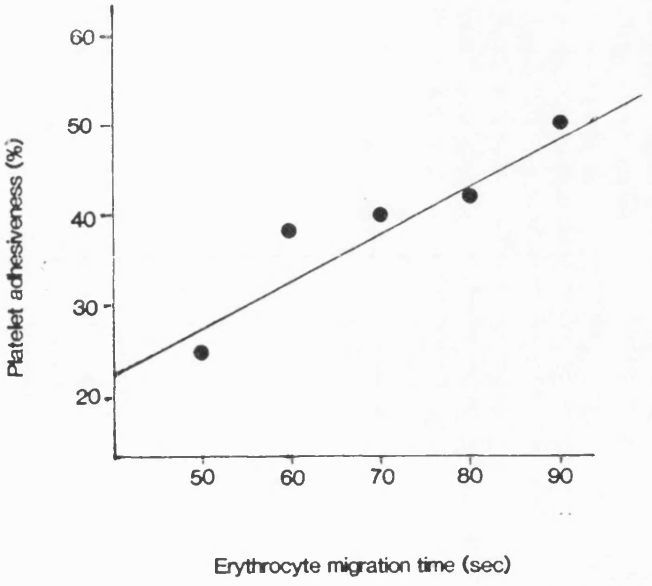
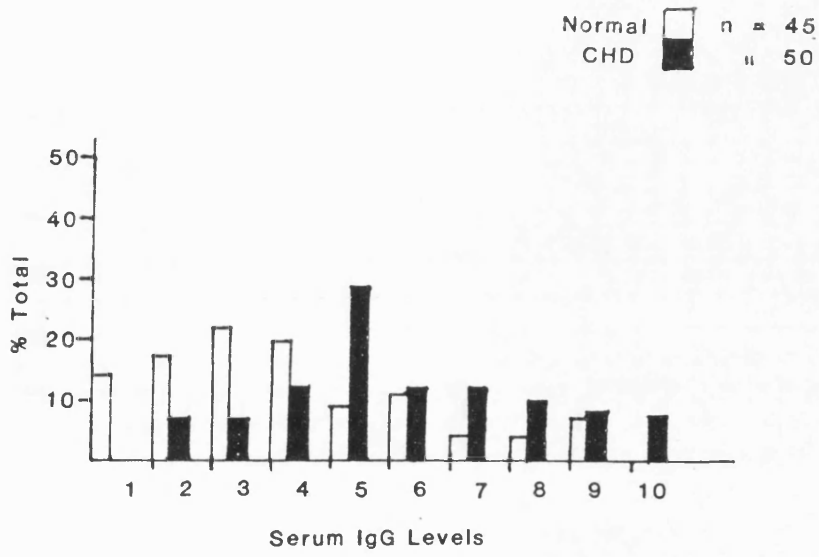


Table 7 Erythrocyte migration time (EMT), and platelet adhesiveness as indices in patients with coronary artery disease.

	EMT (sec)	Platelet adhesiveness (%)
Controls n = 21	Mean = 61.43 S.D. = 7.01	Mean = 29.05 S.D. = 16.37
MI patients n = 28	Mean = 78.14 S.D. = 11.07	Mean = 42.29 S.D. = 15.87
Difference= t =	16.71 6.45	13.24 2.85

From Davies and Lloyd (1967).

Fig 16 Total IgG levels in 45 Control and 50 MI patients.



IgG level	mg/100 ml
1	600 – 699
2	700 – 799
3	800 – 899
4	900 – 999
5	1000 – 1099
6	1100 – 1199
7	1200 – 1299
8	1300 – 1399
9	1400 – 1499
10	1500

Adapted from Davies and Clark (1968).

been implicated in CHD (Annand,1967;Segall,1977;Seely, 1981).

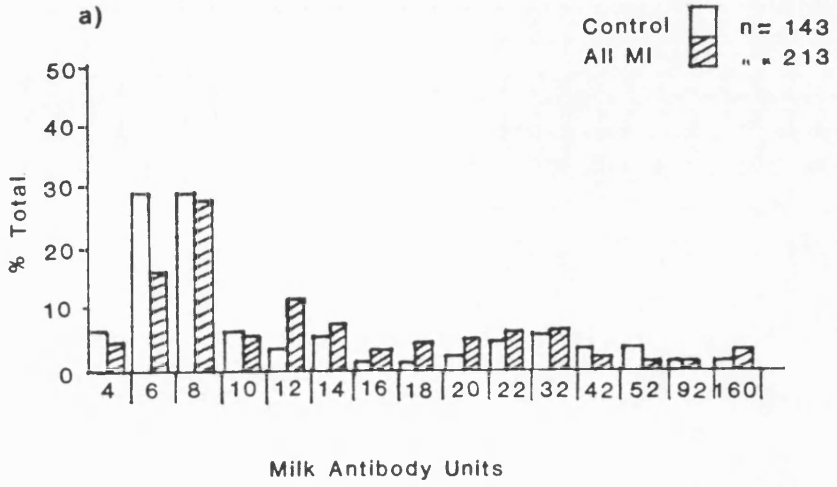
Following this initial report, collaborative studies, with the MRC Epidemiology unit at Cardiff, were initiated. Four comparative matched case-controlled studies were made. The first of these was hospital based and showed a significant difference in antibody levels, to dried cows' milk, between CHD patients and controls. The remaining three community based studies showed that, although the levels of antibodies were significantly raised in CHD patients, the differences were not statistically significant. These community-based studies were on patients who had survived an infarct for up to a year or more and the suspicion arose that that elevated antibody levels were merely sequential to infarction, decreasing with time. However, serial studies on individual patients showed that this was not the case (reviewed by Davies,1984). An alternative explanation was provided in 1971, when the MRC analysis of the data suggested that patients with higher antibody levels were dying earlier; a factor that would tend to decrease average antibody levels in the community-based patients.

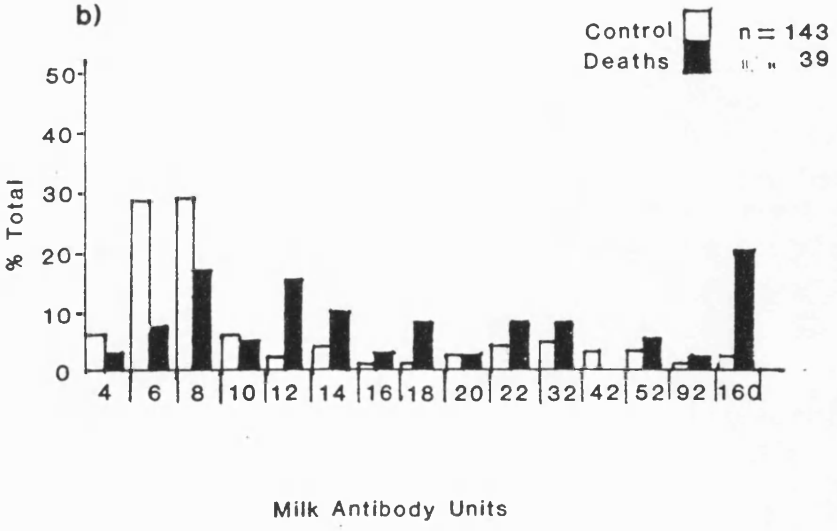
A further paper published in the Lancet (Davies et al,1974) showed conclusively that antibodies to dried cows' milk in CHD patients were raised compared to an age-matched control group (see Fig 17,p45). The difference was much more striking when the levels were analysed from patients who had died within six months, compared with those who had survived.

Fig 17 Comparisons of milk antibody titres in ;

a) Controls and MI patients, and

b) Controls and MI patients who have died within 6-months of infarct.



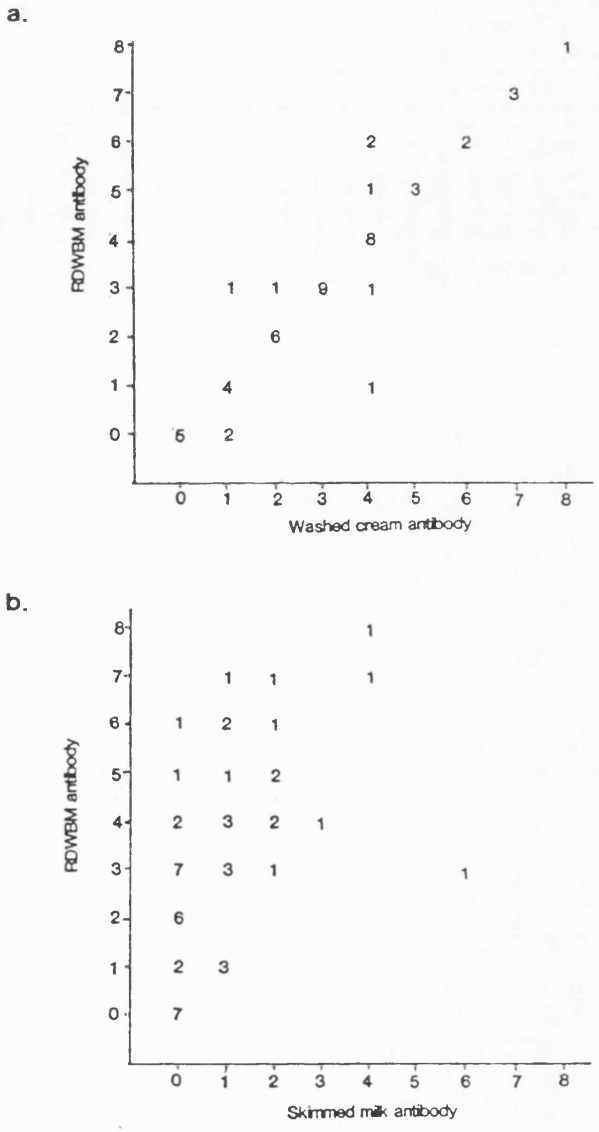


Adapted from Davies et al (1974).

Up to this the point, work had been concerned with dried whole cows' milk and it was clearly essential to determine the individual milk antigens responsible for eliciting the antibody response. The first steps in this direction were reported, in 1980, by Davies et al, who showed an excellent correlation between anti-(whole dried milk) antibodies and anti-(washed cream), antibodies, but not with anti-(skimmed milk antibodies), see Fig 18, p47. It appeared, therefore, that the antibodies of interest were directed against some component of the cream and these studies were extended to show that Bovine Milk-Fat Globule Membrane (BMFGM- see sect 2, p52) was, in fact the relevant antigen (Davies et al,1982; Rees 1985).

Thus, by the early 1980's, Davies had established a link between BMFGM antibody and CHD. Rees and coworkers then proceeded to examine more closely why this antibody, in particular, might be implicated. On the premise that common antigens may be shared by membranes of diverse cells and, in view of the generally accepted importance of platelets in CHD, they decided to examine the possibility that anti-BMFGM antibody might cross-react with human platelets. Antibodies to a preparation of BMFGM were raised in laboratory rabbits and linked to a fluorescein probe. Cross-reaction with human platelets was demonstrated by marked platelet-membrane bound fluorescence of the post-immune sera, but not the pre-immune sera (Davies 1984, Rees, 1985). Platelet clumping

Fig Comparisons of reconstituted dried whole bovine milk (RDWBM) antibody with; a) washed cream antibody titres and b) skimmed milk antibody titres.



From Davies et al (1980).

and aggregation were also marked with post-immune sera but not with pre-immune sera. Davies and co-workers concluded that anti-BMFGM antibody could be atherogenic because of its anti-platelet activity (Davies et al,1982).

Rees has further discussed this connection. He noted that a higher percentage of MI patients, compared to normals, had an excess of active platelets in their blood. Moreover, platelets which have been newly formed in the blood, have higher amounts of vasoactive amines and ADP and so aggregate more readily. Rees hypothesised that BMFGM antibody bound to platelets caused their removal from the circulation, by the reticulo-endothelial system. Such a reaction would be followed by an increased platelet turnover, resulting in a greater proportion of newly formed active platelets (Rees, 1985).

Davies thus proposed that anti-BMFGM antibody was atherogenic, and the fact that it could be demonstrated in the umbilical cord bloods of newly born babies led him to suggest that it may also be pathogenic in cot-deaths and still-births (Davies, 1984).

1.5 Literature Response To Davies' Hypothesis.

The paper published by Davies et al in the Lancet (1974) aroused a great deal of interest. Initial reaction was enthusiastic. Ellis (1974) suggested that Davies' findings could possibly be the most important

nutritional discovery of the century. He went on to propose that the milk of the mother was the best food for an infant, and if not, then a vegetable based infant formula may be a better source of food.

Davies' results were confirmed by Oster et al, (1974) who, like Davies et al, used the tanned red blood cell haemagglutination assay (Boyden,1951; Rees,1973) for anti-cows' milk antibodies. Oster and coworkers confirmed that antibody levels were raised in patients with atherosclerotic disease. They also measured the antibody response to Xanthine Oxidase (XO), a major protein of the BMFGM and again found a statistically significant elevation of antibodies to XO in those subjects with demonstratable atherosclerotic manifestations. In fact, there appeared to be better correlation with antibodies to XO than to cows' milk. Oster's paper was used by him to support his theory that bovine milk XO was a causative factor in CHD. This hypothesis was the subject of a report by the Division of Nutrition, Bureau of foods, Food and Drug Administration (FDA), Washington, D.C. (Carr et al, 1975)

Following Oster's confirmatory report, three papers (Toivanen et al,1975a, Scott et al,1976, Gibney et al,1980) were published all of which failed to show statistical difference in milk antibody levels between MI patients and controls. Toivanen et al, compared IgG and IgM anti-cows' milk antibodies of MI and control patients by using a radioimmunoassay, they reported no

difference between MI and control patients. Their data are, however, confused. Titres presented in the Table of their paper, show average antibody levels for all sera within each group, while a separate set of average titres in their Text exclude sera from samples showing zero antibody levels. The mean levels shown in the text are accordingly raised compared to those of the Table. However, when values are calculated for the Table from the text and vice versa, there are wide discrepancies (see Table 8, p51). Poston (1975) comments upon this fact and states that the figures of the text concerning IgM actually support the Davies' hypothesis. In a reply to Poston, Toivanen et al (1975b), reiterated their conclusion that there was no difference between MI and control patients, and if anything, that IgM was higher in the controls, when all sera were examined.

Davies et al, (1980) offered a further comment on the equivocal results of Toivanen et al (1975a). Davies and co-workers compared levels of antibodies to re-constituted dried whole bovine milk (RDWBM), to washed fresh bovine cream and to skimmed bovine milk (see Fig 18, p47. Anti-RDWBM antibody levels correlated with anti-cream but not with anti-skim milk antibody levels. They pointed out that the data of Toivanen et al were obtained with fat free milk whereas those of Davies and co-workers themselves and those of Oster et al (1974) were obtained by using RDWBM as antigen. Scott et al (1976) who also refuted Davies conclusions used "National Dried Milk" as antigen. This was

Table 8 Discrepancies in the results of Toivanen et al, (1975a).

TEXT

	CONTROL		MI	
IgG	[45]	7.38 (8.30)	[38]	8.46 (8.46)
IgM	[42]	3.20 (3.30)	[40]	3.88 (2.44)

Figs in [ ] = no. of patients

Figs in ( ) = theoretical values for text calculated from Table.

TABLE

	CONTROL		MI	
IgG	[50]	6.64 (5.90)	[47]	6.84 (6.84)
IgM	[50]	2.62 (2.56)	[47]	2.18 (3.47)

Figs in [ ] = no. of patients

Figs in ( ) = theoretical values for Table calculated from Text.

Table 9 A summary of the literature response to Davies' hypothesis.

AUTHORS	Total No MI samples			Total No Control samples		
	Male	Female	Total	Male	Female	Total
Davies et al	213	0	213	143	0	143
Oster et al	25	9	34	22	19	41
Toivanen et al	47	0	47	50	0	50
Scott et al	90	0	90	36	0	36
Gibney et al	33	4	37	25	12	37
	Assay used			**Antigen source**		
Davies et al	Haemagglutination			RDWBM		
Oster et al	"			"		
Toivanen et al	Radioimmunoassay			Fat-Free Dried Milk		
Scott et al	Haemagglutination			National Dried Milk		
Gibney et al	Haemagglutination + ELISA			Spray Dried Milk		
	Support for the Davies' Hypothesis					
Davies et al	+					
Oster et al	+					
Toivanen et al	-					
Scott et al	-					
Gibney et al	-					

unfortunately withdrawn in 1977 and, while, it was known to differ from other infant formulas, was not available for further study. Gibney et al, (1980), the third dissenting group, used a spray dried milk, source unknown.

The results of these surveys are summarised in Table 9, p52.

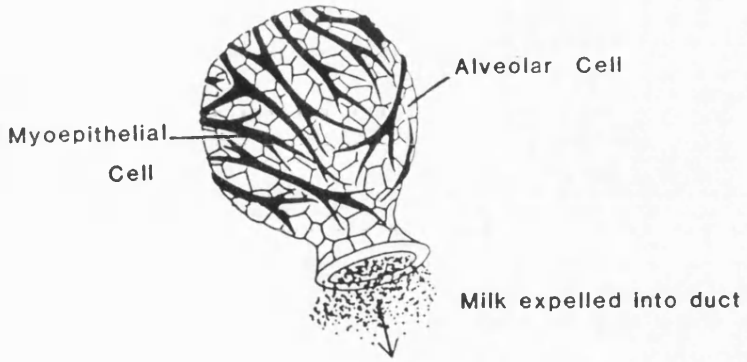
Not withstanding the subsequent discussions, the result of the refuting publications was to effectively discredit the Davies hypothesis; a situation that has remained largely unchanged until now.

2.0 THE BOVINE MILK FAT GLOBULE MEMBRANE (BMFGM).

2.1 Introduction

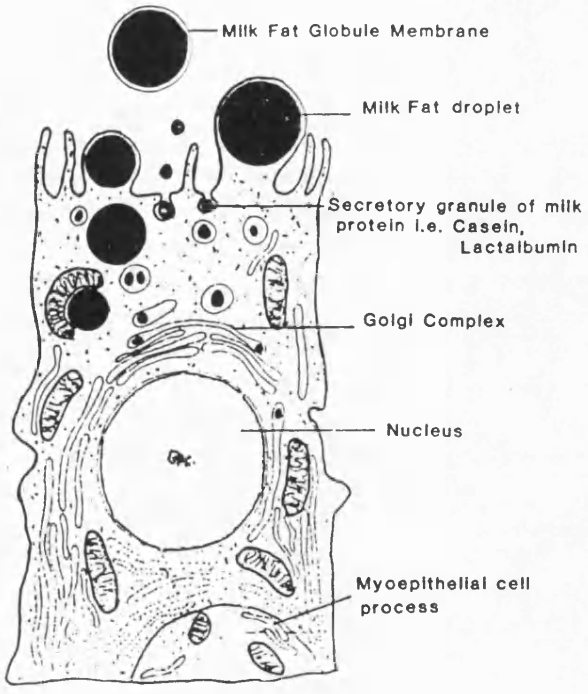
During lactation, milk fats are secreted from mammary epithelial cells into the mammary gland lumen. They are in the form of globules, with diameters ranging from 0.1 - 20µm (Walstra,1966), surrounded by a surface layer of membrane, the BMFGM (see Figs 19,20, p54). At least 98% of the core of each globule is composed of triglycerides, 60% of which is phosphatidylethanolamine and phosphatidylserine (Patton and Keenan,1975). Immediately surrounding this is a layer of materials which appears to be absorbed from the cell prior to secretion, and may, in part, be derived from the endoplasmic reticulum of the cytoplasm. Finally, the

Fig 19 A milk secreting alveolus.



From Alberts et al (1983), p917.

Fig 20 Milk protein and fat production from a milk secreting cell, showing formation of the BMFGM.



From Bloom and Fawcett (1975).

apical membrane of the secretory mammary cell (see Fig 20,p54). During the secretion process, fat droplets within the mammary secretory cells approach the apical membrane, and are progressively enveloped by it before being expelled into the gland lumen. Some of the BMFGM may also be acquired directly from the Golgi apparatus within the cell, but the extent of this contribution remains controversial (Keenan et al,1970; Wooding 1971, 1973; Keenan and Huang,1972; Matel et al,1973;Keenan, 1974; Powell et al,1977). Nevertheless it is generally agreed that the BMFGM is largely derived from the plasma membrane; a fact supported by biochemical analyses (Keenan et al,1970).

Table 10, p56, gives an historical perspective of the discovery of the membrane, the overall composition of which is shown in Table 11, p57.

2.2 BMFGM Lipids

The lipidic fractions of the BMFGM were first characterised by Thompson et al (1961) who showed that individual phospholipids were similar to those of the plasma membrane. There is however, some contamination of the BMFGM by triglycerides of the inner core (Keenan and Huang,1972; Newman and Harrison,1973; Walstra,1974). Interestingly, various gangliosides of BMFGM, presumably derived from the plasma membrane are known to act as receptors for bacterial adhesion and may act to prevent infection of the newborn (Huang,1973; Keenan,1974; Laegrid et al,1986).

Table 10 Major contributions to BMFGM Study

AUTHOR	CONTRIBUTION
Van Leeuwen hoek,1674	Discovered fat globules in milk
Ascherson,1840	Emulsion stabilising substance around globules
Palmer and Samuelson,1924	First to isolate and partially characterise the membrane
King,1955	Summarised all evidence to propose a two-layer membrane structure
Bargmann and Knoop,1959	Showed progressive emergence of BMFGM from apical cell membrane by electron microscopy

Table 11 Gross composition of BMFGM

Constituent	Amount
Proteins	25 to 60% of dry weight
Total lipids	0.5 to 1.1 mg/mg protein
Neutral lipids	56 to 80% of total lipids
Hydrocarbons	1.2% of total lipids
Sterols	0.2 to 5.2% of total lipids
Sterol esters	0.1 to 0.8% of total lipids
Glycerides	53 to 74% of total lipids
Free fatty acids	0.6 to 6.3% of total lipids
Cerebrosides	3.5 nmol/mg protein
Gangliosides	6 nmol/mg protein
Sialic acids	63 nmol/mg protein
Hexoses	0.6 µmol/mg protein
Hexosamines	0.3 µmol/mg protein

From Patton and Keenan (1975).

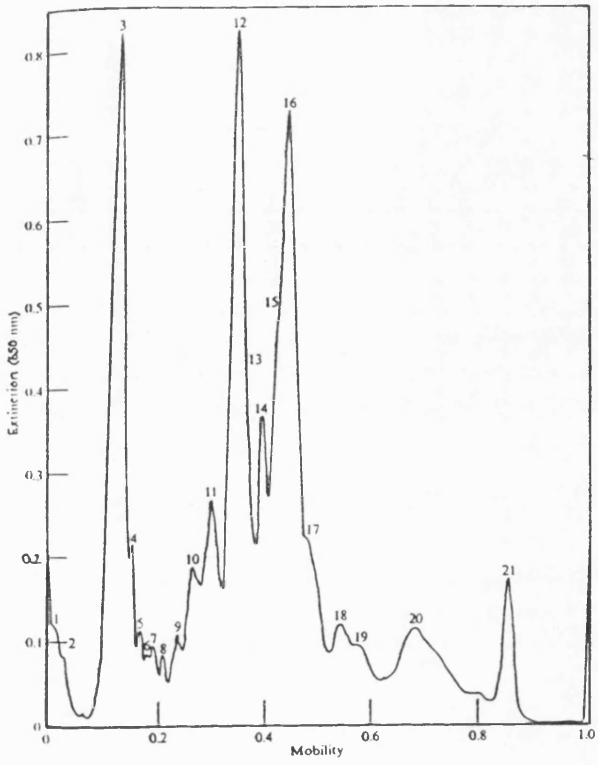
2.3 BMFGM Proteins

Separation of the proteins of the BMFGM by SDS-PAGE leads to the resolution of 8-14 major components (Anderson and Cheeseman,1971; Keenan and Huang,1972; and Kobylka and Carraway,1973). Over 40 polypeptides can be identified after electrofocusing in the presence of urea (Mather,1978). Fig 21, p59, shows a typical SDS-PAGE profile of the membrane, and the molecular weights of the major proteins. The apparent protein composition of the membrane does however depend upon the method of preparation (Basch et al,1985) and molecular weights of the bands vary slightly from paper to paper (Anderson et al,1974).

In all surveys it is clear that two proteins Xanthine Oxidase and Butyrophilin account for nearly 50% of the total Coomassie staining of the membrane:-

Xanthine Oxidase (Mr 150-155KD) costitutes some 8-10% of the BMFGM protein (Morton,1954; Zittle et al, 1956; Briley and Eisenthal,1974) and its degradation products (e.g. with Mr 43KD and 90KD (Mather et al,1982)) may also be detected. Some XO may also occur in mitochondria and microsomes (Dowben et al,1967). The function of XO in the membrane is unknown, but roles have been proposed for it in microbial defence, and vesicle formation(Jarasch et al,1981).

Fig 21 Separation of BMFGM proteins on SDS-polyacrylamide gel.



Apparent molecular weights of the BMFGM proteins shown above:-

3. 155kD
4. 138kD
9. 98kD
10. 89kD
11. 74kD
12. 62.5kD
14. 51.5kD
15. 48kD
16. 43.5kD
17. 37.5kD
18. 31kD
19. 27kD
20. 17.5kD
21. 11kD

From Mather and Keenan (1975)

Butyrophilin (Mr 60-65 KD) is an acidic glycoprotein forming a major part of the dense, fuzzy coat of the fat globule. Immunolocalisation shows it to be present exclusively at the apical, and not the basolateral surface of the mammary epithelial cell (Franke et al,1981). It is suggested that this protein provides a specific marker for the apical surface of the milk secreting cell and its vectorial discharge of lipid globules.

Other proteins known to be present in the membrane include; HLA-Dr antigens (Wiman et al,1979), Acid and Alkaline Phosphatases (Dowben et al,1967), Mg^{2+}-activated ATPase (Nielson and Bjerrum,1977), 5'-Nucleotidase (Patton and Trams, 1971, Huang and Keenan,1972), a glycoprotein PAS IV (Greenwalt et al,1985) and a b-type cytochrome (Jarasch et al,1977).

3.0 ANTIGEN ABSORPTION

Inherent in the Davies' hypothesis is the concept that the antigen, BMFGM, must be present in the blood, to generate an immune response. Many common dietary macromolecules (particularly proteins) can be antigenic. According to Wiseman (1964) nearly all ingested protein is absorbed rapidly and efficiently from the gut lumen following the action of gastric and pancreatic enzymes.

However complete breakdown of protein into amino acids requires about 200 hours (Fisher,1954), and it is likely that most proteins reach the intestinal mucosa in the form of both oligopeptides and amino acids (Silk,1974). Amino acids are taken up in the ileum by specific transport mechanisms. Oligopeptides may bind to specific receptors (secretory IgA molecules (s.IgA)) and be degraded by brush border amino peptidases (Underdown and Schiff,1986). Alternatively, they may be taken into vacuoles for intracellular hydrolysis. Should any oligopeptide escape degradation, it is possible that it may be antigenic as 3-6 amino acids are the optimal size for antigenic determinants (Kabat,1968).

In the fetal and neonatal intestine there is a maturational delay of the gut mucosa, rendering it more permeable to food antigens than in later life (Eastham et al,1978; Rothberg,1969; Walker 1985). Studies with fetuses have shown uptake of large molecules by intestinal epithelial cells (Lev and Orlic,1973; Moxey and Thier,1975), and the neonatal small intestine has a capacity to ingest macromolecules and possibly even cells (Weiler et al,1983) by an endocytotic mechanism. this is of particular importance in animal species that depend, for their passive immunity, on maternally transmitted immunoglobulins via colostrum during the first few days after birth (Clark,1959). The phenomenon, known as "closure" is the result of intestinal maturational events i.e. the glycocalyx development,

which is facilitated by colostrum itself (Heird and Hansen,1977).

Despite the commonly held notion that the adult gut is an impenetrable barrier to the uptake of luminal antigens, experimental and clinical evidence suggests that this is not so. Macromolecules may be absorbed in healthy adults, not in sufficient quantities to be of nutritional importance, but, nevertheless, enough (up to 2% of ingested protein) to be antigenically or biologically active (Wilson and Walzer,1935; Lippard et al,1936; Walker and Isselbacher,1974). The absorption of antigen in healthy adults was first documented at the beginning of the century by Uhlenhuth (1900) who reported the formation of circulating antibodies in rabbits fed on ovalbumin. Korenblat and co-workers (1968) demonstrated precipitins in some humans after ingesting milk, and Paganelli et al (1979) showed that consumption of high levels of milk, in a single feed, was followed by a transient rise in levels of serum immune complexes. These subsequently fell to levels below those normally associated with tissue damage. It is possible that the immune system, in a novel role, is aiding the removal, from the bloodstream of small quantities of undigested or foreign protein (Hamburger et al,1987). Certain disease states, such as Ulcerative Colitis (Jewell and Truelove,1972), gastro-intestinal allergy, inflammatory bowel disease (Ferguson,1977), Coeliacs disease (Shiner and Ballard,1972) and some

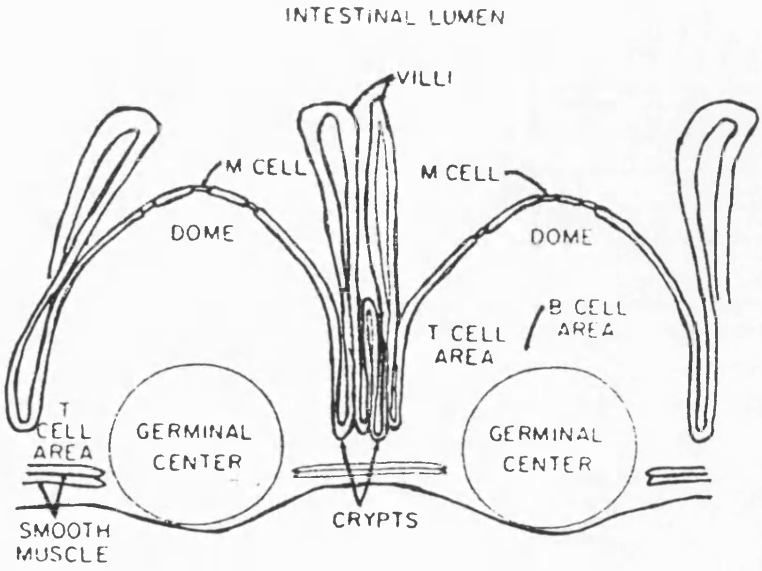
immune complex mediated disease (Walker,1975) may be caused by absorption of intraluminal antigens, particularly food derived, resulting in the triggering of allergic type responses (Hutchins and Walker Smith, 1982).

Antigen absorption in the healthy individual can occur at three levels:-

1. The M-cell is a specialised epithelial cell, overlying the gut-associated lymphoid tissue (GALT) in the ileum. The whole comprises the Peyer's Patches (see Fig 22, p64). M-cells are specially adapted for antigen transport (LeFevre and Joel,1984), and delivery to subepithelial lymphocytes and are known to be penetrated by various colloids and protein antigens (Joel et al,1970; Owen,1977). It is not known whether these cells act merely to "sample" antigens present in the gut lumen or whether they may also process them, as do macrophages (Richman et al,1981).

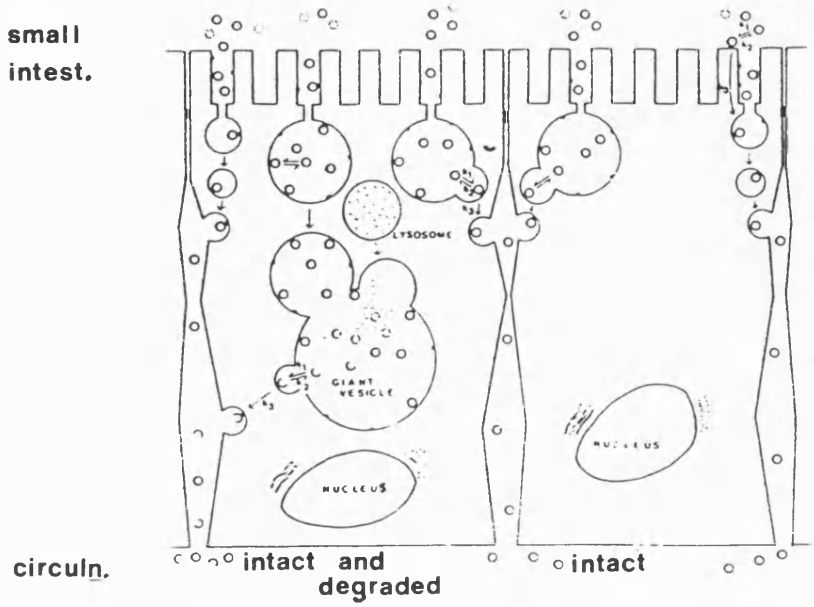
2. The normal epithelial cell, as already mentioned, possesses receptors for oligopeptides (s.IgA). However, they also seem to possess a non-specific uptake mechanism whereby molecules are adsorbed on their luminal surface and endocytosed. The resulting small intracellular vesicles fuse to form phagosomes, which fuse further with lysosomes so effecting intracellular digestion (see Fig 23, p64). This may represent the

Fig 22 Diagrammatic representation of a Peyers' patch, consisting of two follicles (the M-cells are enlarged for emphasis).



From LeFevre and Joel (1984).

Fig 23 Possible routes of transmission of intact and degraded protein, from the small intestine into the circulation.



From Hemmings (1978).

residual of the primitive absorptive mechanism found in the gut of the neonate. Undigested or partly digested breakdown products may be released into the interstitial space, where they are presented to the subepithelial immune system or transported in immune-complex form to the liver (Walker,1978).

Histochemical studies have shown that small quantities of antigen are uptaken preferentially by M-cells, but larger quantities by epithelial cells (Walker,1979). Various factors influence particle uptake by cells. Particles more hydrophobic than the surface of the cell are selectively phagocytosed (LeFevre and Joel,1984). This may be of relevance when considering uptake of BMFGM. Homogenisation of milk breaks up fat globules from an average diameter of 3.5μm to 1μm and increases the number of globules 100-fold. Ross et al, (1980), have compared the uptake of such globules to that of liposomes which are used for transporting therapeutic substances across the intestinal barrier (Gregoriadus,1976).

3. Intra-cellular junctions can be widened in certain disease states i.e. inflammation or surgical intervention of the gut. This can result in enhanced uptake of luminal antigen (Rhodes and Kornovsky,1971; Reinhardt et al,1983).

The normal consequences of antigen absorption involve local production, in the intestine, of s.IgA which acts

to exclude dietary antigens (Walker and Isselbacher, 1977). In patients with selective IgA deficiency, uptake of bovine antigens is increased (Cunningham-Rundles et al,1978). A systemic immune response may occasionally arise, but, most commonly, a state of tolerance develops determined principally by T-cell unresponsiveness (Challacombe and Tomasi,1980; Mowat,1987).

AIMS

The major aims of this thesis were as follows:-

1. To verify and expand on the observation of Davies, that anti-milk antibodies are elevated in MI patients. However, anti-BMFGM, (rather than anti-milk), antibodies are measured specifically by means of an ELISA, developed for routine assay.

2. To investigate, in more detail, the antigenic determinants of the BMFGM itself.

3. To establish whether or not a true cross-reaction exists between the BMFGM and the platelet membrane.

MATERIALS AND METHODS

MATERIALS

1.1 Chemicals and reagents

Column resins and immunochemicals were provided by the Sigma Chemical Company (Poole, Dorset, U.K.), unless otherwise stated.

All buffering reagents were supplied by either Fisons plc. (Loughborough, Leics., U.K.) or BDH Chemicals Ltd. (Poole, Dorset, U.K.). Phosphate buffered saline (PBS) was supplied in tablet form by Oxoid Ltd (Basingstoke, Hants, U.K.).

Chemicals for SDS-PAGE gel electrophoresis were supplied by BDH Chemicals Ltd. (Poole, Dorset, U.K.), and were of the Electran range.

All other chemicals were provided by Sigma Chemical Company (Poole, Dorset, U.K.) or BDH Chemicals Ltd. (Poole, Dorset,U.K.).

1.2 Serum samples

Normal rabbit serum and normal goat serum were provided by Sera-Lab Ltd (Crawley Down, Sussex, U.K.).

Human serum samples came from the following sources.

1. Control samples for MI samples were provided by the MRC Epidemiology Unit in Cardiff. The samples were from males of age 49-64 and were collected from Caerphilly (Wales, U.K.) mainly, and Speedwell (Bristol U.K.) as part of a survey, examining known risk factors for Heart Disease (Caerphilly and Speedwell Collaborative group, 1984).

2. MI samples were provided by the same MRC unit, as part of a survey examining diet in secondary prevention of MI. These cases came from areas all over South Wales (Swansea to Newport), with a few from Gloucester and Bristol, and were males of ages 45-80.

3. Myasthenic sera had been collected from the Royal United Hospital (Bath, Avon, U.K.), the Royal Infirmary and Southmead (Bristol, Avon, U.K.). Samples were stored at -40°C.

4. Serum samples from Motor Neurone Disease sufferers was provided by Charing Cross Hospital (London, U.K.), as were those from migraine sufferers sera (provided by Dr.P. Davies).

5. Immune Thrombocytopenic sera were provided by the Haematology Department of the West Wales Hospital (Carmarthen, Wales, U.K.), and had been characterised by positive immunofluorescence on Megakaryocytes.

6. All other human serum was provided by the South West Regional Blood Transfusion Service. This serum was from unknown healthy donors and was generally pooled to prepare a Standard serum.

METHODS

2.0 PREPARATIVE PROCEDURES

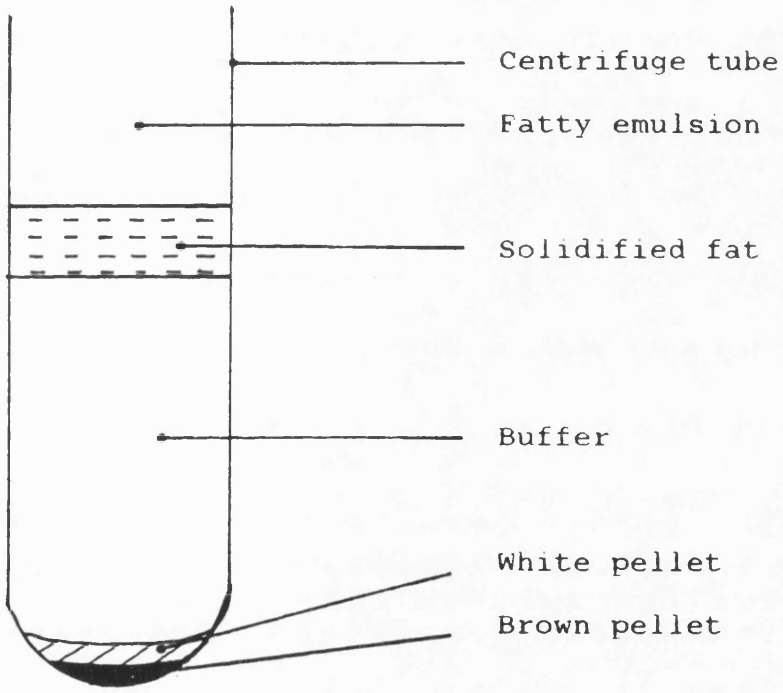
2.1 Preparation of BMFGM

The method was followed according to Mather and Keenan (1975), except for the inclusion of protease inhibitors during the washing stages of cream. Their inclusion was found to give clearer SDS-PAGE profiles of BMFGM.

Cream was separated from fresh uncooled milk (12 L), from Friesian cows in mid-lactation, kindly provided by Bassett farm (Claverton Down, Bath, U.K.). Milk (12L) was centrifuged (170g,15min), at 37°C and the fat globules were washed with an equal volume of distilled water (37°C), containing 0.15mM-phenylmethylsulfonyl fluoride (PMSF), 10m-ethylenediaminetetraacetic acid, disodium salt (EDTA), and 2mM-benzamidine, before centrifugation at 37°C (250g,10min). The washing of the cream was repeated 3-4 times until a clear wash was obtained and the final wash was performed at 4°C. The cream was resuspended (5:1 v/v) in 50mM-Tris/HCl, pH7.5 and chilled to 4°C in an ice-bath before churning to butter in a Waring blender (5-10 min). The butter was collected and liquified at 37°C, in order to release trapped BMFGM. This was further separated from the fatty mixture by centrifugation (100,000g,1h) at 20°C.

The brown-white pellet of BMFGM was collected (see Fig **24**) and stored at -80°C. Yields varied from 2-6g wet weight of BMFGM from 12L of milk, depending upon the time of year.

Fig **24** BMFGM purification, after centrifugation of crude extract at 100,000g.



Brown + White pellets = BMFGM

2.2 Preparation of platelet membrane.

Platelet Rich plasma (PRP) was obtained from the South-West Regional blood transfusion service. Blood group "O" platelets were used in order to minimise cross-reaction of sera with membrane antigens.

(i) Preparation according to Winiarski and Ekelund (1986), modified from Kaser-Glanzmann et al, (1977).

PRP was centrifuged (1300g,10min), and the platelet pellet was resuspended in PBS buffer containing 0.33% EDTA, pH 7.4. The pellet was washed three times by centrifugation (1300g,10min) and was then resuspended in EDTA-PBS. The platelets were adjusted to a concentration of 10^9/ml (counted by means of an improved Neubauer counting chamber) and sonicated (Model 180 sonicator, from Ultrasonics Ltd., Shipley, Yorks., U.K.) in 25ml batches, surrounded by ice. 100W was delivered in 2 x 30s bursts with a 30s interval during which the probe was retuned, within the sample. Batches of sonicated platelets were pooled and centrifuged (10,000g,15min) at 4°C. The pellet was discarded and the supernatant was collected and recentrifuged (100,000g,1h) at 4°C. The pellet, containing platelet membrane, was resuspended by homogenisation in EDTA-PBS and stored at -80°C.

(ii) Platelet membrane preparation according to Barber and Jamieson (1970).

PRP was centrifuged (1300g,10min) and the pellet was resuspended in 0.01M-Tris/HCL, pH7.5, containing 1mM-EDTA, and 0.15M NaCl before recentrifuging as above. In

this way, platelets were washed three times. The intracellular glycerol concentration of the platelets was then raised to 4.3M by centrifuging them (1,465g,30min followed by 5,860g,10min) through an isotonic glycerol gradient (0-40%) at 4°C. The supernatant glycerol solutions were removed and Tris-sucrose buffer (Washing buffer above containing 0.25M sucrose) was added to the remaining platelet pellet (5:1 v/v), which was immediately vortexed. The lysed platelet suspension was layered onto a sucrose solution (27% w/v) and centrifuged (25,000g,5h) at 4°C, when subcellular components and debris sedimented to the bottom of the tubes and the membrane fraction remained as a narrow band above the sucrose interface. The membranes were isolated, diluted in cold Tris-sucrose buffer (1:10 v/v) and centrifuged (106,000g,1h) at 4°C. The membrane pellet was resuspended in cold Tris-sucrose buffer, layered on a linear sucrose density gradient (15-40% w/v) and centrifuged (63,500g,18h) at 4°C. Two membrane bands were separated, diluted (1:10 v/v) in cold Tris-sucrose buffer and centrifuged (106,000g,1h) at 4°C. Platelet membrane was resuspended, by homogenisation in Tris-sucrose buffer, and stored at -80°C.

Membrane protein, for both procedures, was estimated by using the SDS-Lowry method (Methods Sect 3.1 (ii), p**85**).

2.3 Preparation of washed platelets.

The protocol of Tangen et al (1970) was followed.

(i) Centrifugation

PRP was centrifuged (1,300g,10min), and the platelet pellet was resuspended in 0.154M-Tris buffer, pH 7.4 (5 x the original volume) containing 0.154M NaCl, and 0.077M EDTA before centrifuging as before. This process of washing the platelets was repeated twice. The platelet pellet was finally resuspended in 5mM-Tris/HCl, pH 7.4, containing 0.154M NaCl, 50μM $CaCL_2$ and 1% w/v glucose.

(ii) Gel filtration

A silanised glass column (1.5 x 30cm) was prepared containing Sepharose 2B-300 (200ml) (Sigma Chemical Company, Poole, U.K.) in the above buffer. A slurry was poured into the column and allowed to settle overnight, under gravity, before washing with the same buffer (2.5 bed volumes). A sample of PRP (1-5ml) was layered on top of the gel, under buffer, and was allowed to flow through at 25ml/h. Platelet peaks (detected visually, or by A_{280}) emerged well ahead of the plasma peak and were collected in silanised tubes, pooled, and counted.

2.4 Preparation of Bovine Xanthine Oxidase.

(i) The method according to Nakamura and Yamazaki (1982) was followed.

Buffer B - 0.2M-K_2HPO_4, containing 2.5mM-DTT, 1mM-EDTA, and 1.25mM-salicylate.

Buffer A - As buffer B, but using 0.2M-Na_2HPO_4 in place of the potassium salt.

2.5mM-DTT, 1mM-EDTA, and 1.25mM-salicylate were added to fresh milk (9L), and cooled to 4°C. All further steps were carried out in a cold room at 4°C. The solution was centrifuged (3,000g,30min) and an equal volume of Buffer B was added to the supernatant followed by slow addition of cold butanol (-20°C),(15% v/v), and 15% w/v ammonium sulphate. The resulting slurry, was stirred for 1hr, removing all lumps of cream, before centrifuging (13,000g,20min). 15% Ammonium sulphate (w/v) was added slowly to the lower aqueous phase and the solution was stirred for 30min then left to separate for 2h. The upper phase was centrifuged (10,000g,30min) and Buffer A was added to the supernatant, to give a final volume of approximately 30ml, which was dialysed overnight against a large volume of Buffer A. The dialysate was centrifuged (20,000g,1h) and applied to a calcium phosphate column, prepared as follows.

Calcium phosphate gel was suspended in Buffer A (1:4 v/v), washed five times in the same buffer, packed into a column (3cm x 25cm) and washed with Buffer A (2.5 x bed volume).

The dialysate was applied to the column, eluted (10ml/h) with Buffer A and collected (7.5 ml fractions), until A_{280} returned to $\leq$ 0.005. Bound XO was eluted from the column by Buffer A containing 5% ammonium sulphate. XO was dialysed overnight at 4°C, into a suitable buffer, to remove ammonium sulphate.

(ii) Purification by Molecular Exclusion.

A Sephacryl G-200 column (2cm x 1m) was used in an

attempt to further purify XO as prepared from the calcium phosphate method (See Results Sect 5.3, p[167]). XO mixture (20-25 mg), dialysed into running buffer was applied to the column and allowed to elute through overnight (40ml/hr) in 7.5mM-KH_2PO_4/20mM-Na_2HPO_4, pH 7.3. Fractions (10ml) were collected and assayed for protein at 280nm.

(iii) The method according to Nishino et al (1981).

AH-Sepharose 4B folate was a gift from Dr Bray, Department of Molecular Sciences, University of Sussex, U.K. The general procedure for covalently coupling AH-Sepharose 4B with folate is described by Nishino et al, (1981). The column matrix was stored in the dark, with 0.02% azide when not in use. The gel was poured into a column (1.5cm x 9cm), covered in aluminium foil and allowed to settle overnight, under gravity.

Buffer A contained 0.1M-sodium pyrophosphate buffer, pH8.5, (20%) and 0.05M-Tris/HCl, pH7.8 (80%), together with 0.2mM-EDTA.

Buffer B contained 0.1M-sodium pyrophosphate buffer, pH8.5, (30%), and 0.05M-Tris/HCl, pH7.8, (70%), containing 0.2mM-EDTA and 0.05mM-hypoxanthine (final concentrations).

The folate column eluted with buffer A (2.5 x bed vol) and XO (prepared from method (i) above, was loaded onto the column (column binding capacity 3-4 mg protein/ml gel). The sample was eluted with buffer A at 25ml/hr and collected in 5 ml fractions,until no absorbance at A_{280} was detected. Bound XO was then eluted with buffer B and dialysed into a suitable buffer, at 4°C.

2.5 Preparation of IgG from serum.

The method according to Johnstone and Thorpe (1982) was followed. All operations were carried out at 25°C. Human serum (usually 5-10 ml) was warmed to 25°C and sodium sulphate was added to make an 18% w/v solution, which was then stirred for 30min and centrifuged (3,000g,30min). The supernatant was discarded and the volume of the precipitate noted. The precipitate was redissolved in distilled water (½ volume of the original serum),and sodium sulphate was added again to make a 14% w/v solution. The solution was stirred for 30 min, then centrifuged as before. The process of sodium sulphate addition, stirring and centrifuging was repeated until a clear supernatant was obtained, when the precipitate was redissolved in distilled water and dialysed against 20mM-sodium phosphate buffer, pH8.

For the preparation of rabbit IgG the procedure above was followed, but the final precipitate was dialysed against 20mM-sodium phosphate, pH 7.5.

For the preparation of goat IgG, serum was treated with 14% w/v sodium sulphate for the initial addition, and the final precipitate was dialysed against 20mM-sodium phosphate buffer, pH 7.5.

The above dialysates were further purified on a DE-52 ion-exchange column.

Pre-swollen DE-52 (diethylaminoethyl cellulose anion exchanger), Whatman Chemical Separation Ltd., (Maidstone, Kent, U.K.) was equilibrated with 20mM-sodium phosphate, pH8 at 20ml/g DE-52. Fines above the slurry were removed

by aspiration and the slurry was washed 3 times with buffer. After each wash, fines were removed and the resulting slurry was stored at 4°C with 0.01% azide, until required.

DE-52 was packed into a column (1.5 x 17cm) and equilibrated with the required phosphate buffer (i.e. the buffer into which the final precipitate had been dialysed). The sample was applied to the column, and IgG was eluted through with equlibrating buffer (30ml/h). Absorbance was monitored at 280nm, and fractions (5ml) were collected. Impurities were subsequently removed by elution with 0.5M NaCl.

2.6 Preparation of $F(ab')_2$, Fab', and Fc fragments from human IgG.

The method according to Hudson and Hay (1976) was followed.

(i) Preparation of $F(ab')^2$ and pFc fragments.

Pepsin digests human IgG into one $F(ab)_2$ fragment and smaller Fc fragments, pFc.

An IgG solution (prepared as described in Methods 2.5, p 77, was concentrated to at least 10mg/ml and dialysed against 0.01M-sodium acetate and the pH of the dialysate was adjusted to pH 4.5 with acetic acid. Pepsin (from porcine stomach mucosa, 2500-3500 units/mg (from Sigma Chemical Company, Poole, Dorset, U.K.)) was added (1mg/50mg IgG) to the dialysate and was left to stir at 37°C for 24h. The solution was then centrifuged (3,000g,15min) and any precipitate formed was discarded.

The pH of the supernatant was adjusted to pH 7.4 and the latter was further dialysed against 10mM-sodium phosphate buffer, pH7.4.

(ii) Preparation of Fab' and Fc fragments.

Papain digests IgG into two Fab' fragments and one Fc fragment.

IgG was concentrated, to at least 10mg/ml, in 20mM-sodium phosphate buffer, pH7.4 and 10mM-cysteine, 2mM-EDTA, and papain (from papaya latex, 16-40 units/mg, (Sigma Chemical Company, Poole, Dorset, U.K.)) were added (1mg/100mg IgG). The solution was stirred at 37°C for 4h (transparency indicated completion of reaction). 20mM-Iodoacetate was added and the solution was left on ice for one hour, then dialysed against sodium phosphate buffer, pH 7.4.

Separation of fragments

a) Protein A affinity chromatography.

$F(ab)_2$ fragments could be separated from undigested IgG and pFc fragments, likewise Fab from IgG and Fc by use of a Protein A column (provided by Dr.A.Jehanli, Bath Univ., Bath,U.K.). Digests were applied to the column and eluted with 10mM-sodium phosphate buffer, pH 7.4. $F(ab)_2$ and Fab fragments pass through unbound, whereas IgG, pFc and Fc fragments, which bind to the column, are eluted with 0.1M-glycine/HCl, pH2.8.

b) Molecular exclusion chromatography.

Digests were applied to a Sephacryl G-200 column (2 x 100cm) and eluted, overnight, in 10mM-sodium phosphate buffer, pH7.4 at a flow rate of 40ml/h. Fractions (10ml)

were collected and assayed (A_{280}) for protein content. Fractions were then pooled (according to MWt) and concentrated i.e. IgG (150kD), $F(ab)_2$ (100kD), Fab and Fc (50kD).

Protein A chromatography does not allow separation of Fc fragments from IgG and Molecular exclusion does not allow separation of Fab and Fc fragments, so a combination of both methods was used.

Fragments were tested for homogeneity by immuno-electrophoresis (see Methods Section 4.3, p 95), then concentrated to at least 1mg/ml and stored at -40°C.

2.7 Preparation of goat anti-(human-IgG) IgG horseradish peroxidase conjugate (IgG-HRP).

Horseradish peroxidase (HRP, 4mg/ml H_2O was stirred for 20 min at room temperature with freshly prepared 0.1M-sodium periodate (0.2ml). The greenish/grey solution was dialysed overnight in 1mM-sodium acetate, pH4.4, and the resulting orange solution was titrated, to pH9.5, with 0.2M-NaOH followed by the immediate addition of goat anti-(human IgG) IgG, [8-10mg in 50mM-sodium carbonate buffer pH9.5, (1ml)]. The goat IgG was prepared from goat anti-serum (provided by Dr.A.Jehanli, Bath Univ.,Bath,U.K.) according to the method described in Methods Sect 2.5, p 77. The solution was stirred at room temperature for 2h followed by the addition of freshly prepared sodium borohydride (4mg/ml in H_2O). The solution was allowed to stand for 2h at 4°C, was then dialysed overnight against PBS.

Unconjugated HRP and IgG were removed by chromatography on a Sephacryl G-200 column (0.75 x 100cm), collecting fractions (7.5ml) at a flow rate of 30ml/h in 20mM sodium phosphate buffer, pH7.4.

The IgG-HRP was concentrated, to 5mg/ml, in equal volumes of PBS and glycerol, and stored at -40°C for further use.

2.8 Preparation of rabbit immune-sera.

Antigen (100µg protein) was made up in sterilised PBS (0.5ml), homogenised if necessary, then mixed with an equal volume of Freunds Complete Adjuvant. Blood (10ml) (for pre-immune sera) was taken from the ears of Californian rabbits, (Animal house, Bath Univ.), which were then injected (2 x 0.5ml) in their hind limbs. Two weeks later the rabbits were given the same injection, but this time antigen was prepared in sterilised PBS alone. The same injection was again delivered one-month later. Approximately 5 days after this final injection, a small sample of blood (0.5ml) was taken from an ear vein and tested for reactivity toward antigen. If positive the animal was then sacrificed, and blood was collected (60-100ml).

Blood was left to clot at 37°C for 1h, then overnight at 4°C. The suspension was centrifuged (30,000g,10min) and the supernatant was collected as serum.

2.9 Affinity purification of anti-BMFGM antibody.

The development of these methods are described in, Results Sect 5.1, p152.

I) Batch absorption

BMFGM was homogenised in human serum (25mg/ml) and left to stir at 37°C for 1h. The suspension was then centrifuged (100,000g, 1h), and the BMFGM pellet was resuspended in PBS, containing 0.75M NaCl incubated at 37°C for 1h and then centrifuged as above. The BMFGM pellet was then resuspended in 0.05M Gly/HCl, pH2.8, (1ml), incubated at 37°C for 1h while stirring, and recentrifuged. The supernatant was decanted, neutralised by the addition of 4M NaOH, dialysed into a large volume of 0.1% ammonium acetate overnight at 4°C and then lyophilised. The affinity-purified antibodies were redissolved in PBS.

II) Preparation of affinity column

Concanavalin A - Sepharose 4B (ConA-Sepharose), (5ml), was washed with PBS (5:1 v/v) three times, separating, each time, by decantation. BMFGM suspended in PBS (5ml,0.1g/ml) was stirred overnight with washed ConA-Sepharose. The slurry was centrifuged (10,000g,10min) and the supernatant, containing unbound BMFGM was removed. The remaining slurry was washed by the addition of PBS (5:1 v/v) and recentrifuged. This process was continued until a clear supernatant was obtained. The slurry, containing BMFGM bound to ConA-Sepharose, was poured into a small column and allowed to settle overnight under gravity. BMFGM was then cross-linked covalently to the column by using Dimethylsuberimidate (DMSI), (Lang et al, 1982). DMSI (0.17-0.2mg/ml) in 0.2M triethanolamine, pH 8.4, was passed through the column (15ml/h) for 3h and the reaction was terminated by recirculating 0.5M lysine

through the column overnight, in the same buffer. The column was then washed with PBS for 2h at 10ml/h, and for 1h at 20ml/hr.

Before the column was used for affinity purification, it was washed with 0.05M ammonium hydroxide, pH11, to remove any non-specifically bound protein from the BMFGM. The column was stored at 4°C with 0.02% azide.

III Affinity purification of anti-BMFGM antibodies using the above column.

Bovine Serum Albumin (BSA), (10mg/ml PBS) was passed, at a flow rate of 15 ml/h, for 1h, through the column which was then washed through with PBS until $A_{280} < 0.005$. IgG, from which anti-BMFGM antibody was to be prepared, was then allowed to circulate through the column overnight at 10ml/h. Unbound IgG was then eluted with PBS (30ml/h; 5ml fractions) until absorbance at A_{280} was negligible. The column was then washed with PBS containing 0.85M-NaCl, 0.2M α-methyl mannoside and 0.2M α-methylglucoside, until the absorbance at A_{280} returned to <0.005. 0.05M-ammonium hydroxide, pH11, was then passed through the column (30ml/h), and the fractions (2ml), containing affinity purified anti-BMFGM antibody, were pooled and immediately neutralised with 2M-acetic acid, dialysed against 0.1% ammonium acetate, and lyophilised.

Antibody was either stored, lyophilised, at -40°C, or made up in PBS (1mg/ml at least) and stored at -40°C.

3.0 ASSAY TECHNIQUES

3.1 Protein estimations

(i) Soluble protein

Method 1

An estimation of protein concentration was made, by measuring the absorbance (280nm) of a suitably diluted protein solution against a diluent blank.

Concentrations of samples were then calculated from their known extinction co-efficients.

For mixtures of unknown proteins or proteins with unknown extinction coefficients, the absorbance of the solution was measured at both 260nm and 280nm (Warburg and Christian, 1940) and the protein concentration was calculated a follows;

$(O.D._{280nm} \times 1.55) - (O.D._{260nm} \times 0.76)$ = mg/ml protein.

Method 2

The method is based on the binding of Coomassie Brilliant Blue dye to proteins, in the presence of ethanol and phosphoric acid (Bradford,1976).

a) Microassay (For samples containing less than or equal to 25µg/ml protein)

Duplicates of samples containing protein were made up in either water or phosphate buffer (0.8ml). To this was added Bio-Rad protein assay dye-reagent (0.2ml), Bio-Rad Laboratories GmbH, (Munich, West Germany) and the solutions were mixed by inversion.

A standard curve was prepared from duplicate samples (1-25µg) of BSA in the corresponding solvent (0.8ml). A standard curve was prepared every time.

The samples containing dye were left for 5-60 min, then read at 595nm.

b) Standard assay (For samples containing 20 - 140µg protein)

Unknown samples were made up as above, but in 0.1ml volumes, and mixed with dye reagent (5ml). A standard curve was prepared by using BSA (0.2-1.4 mg/ml). The procedure was then as for the microassay.

(ii) Membrane protein.

The method used was a modification of the Lowry method (Lowry et al, 1951), futher developed by Markwell et al, (1981) and Carruthers and Melchior (1983).

Solution A - 2% (w/v) Na_2CO_3 in 0.4% (w/v) NaOH, 0.16% (w/v) KNa Tartrate and 3% (w/v) SDS.

Solution B - 4% (w/v) $CuSO_4$.

To unknown samples diluted in water or phosphate buffer (200µL) a mixture of solutions A and B (100:1 v/v), were added (1ml). The resulting solution (1.2ml) was mixed by inversion and left for 10min. Folin and Ciocalteau reagent (1:1 H_2O v/v) was then added at equal volumes to each sample, mixed by inversion and left for 30min. Absorbance was read at 750nm.

A standard curve was prepared from 0-100µg BSA, dissolved in the same buffer as the samples being assayed.

3.2 Assay of XO activity

XO exists in two forms, an oxidase (XO), and a deydrogenase (XD). Inclusion of DTT in buffers favoured

preparation of the dehydrogenase form. Activities were measured according to the method of Nakamura and Yamazaki (1982).

Reaction mixture (2ml) contained:-

70μM Xanthine (first solubilised in a minimal volume of 4M NaOH) and 0.1M-sodium pyrophosphate, pH8.3.

0.5mM-NAD^+ - was additionally present for the Dehydrogenase assay.

A suitable amount of enzyme contained in a volume of no greater than 100μl, was added to reaction mixture (2ml) in a cuvette, and mixed by inversion.

Xanthine Oxidase activity was measured spectrophotometrically by the increase in absorbance at 292nm under aerobic conditions, corresponding to the oxidation of Xanthine to Uric acid.

[A unit of XO activity was measured as the amount of enzyme yielding 1μmole of uric acid/min, E for Uric acid at 292nm is $12.2mM^{-1}cm^{-1}$, (Dawson et al,1974).]

Xanthine Dehydrogenase (XD) activity was measured as the increase in absorbance at 340nm corresponding to the reduction of NAD^+.

[A unit of XD activity is defined as the amount of enzyme yielding 1μmole NADH/min, E for NADH at 340nm = $6.22mM^{-1}cm^{-1}$, (Dawson et al,1974).]

3.3 Enzyme Linked immunosorbent assays (ELISAs)

(I) BMFGM

The development of this ELISA is described in more detail

in Results Sect 1.1, p101.

BMFGM was prepared as described in Methods Sect 2.1 and used to coat microtitre wells. BMFGM was homogenised in 50mM-sodium carbonate buffer, pH9.6 at a concentration of 10µg BMFGM protein /ml carbonate buffer. Suspension (100µl/well) was added to polystyrene microtitre plates, (Flow Laboratories Ltd., Rickmansworth, Herts., U.K.) which were then left to incubate at 4°C overnight or for 3h at 37°C, washed with washing and blocking buffer (200µL/well) (PBS containing 0.05% (v/v) Tween 20), for 3 x 10min. Serum or IgG, diluted appropriately in PBS/Tween, was added (100µl/well) and left to incubate overnight at 4°C, or for 1h at 37°C. Wells were then washed as before and conjugate was added (1:1000 in PBS/Tween, 100µl/well) and was allowed to incubate at room temperature for 1-2h. Wells were then washed as before and stain was applied. 0.01M-sodium acetate/citrate buffer, pH6 containing 0.3mM-H_2O_2 and 420µM-tetramethyl benzidine (TMB) was added (100µl/well) and allowed to incubate at room temperature until a suitable colour developed, as judged by eye. The reaction was then stopped by the addition of 2M-H_2SO_4, (50µl/well). The absorbance (450nm) was read by using a Titertek Multiskan MCC (Flow Laboratories, Rickmansworth, U.K.).

For human sera or IgG, conjugate was either laboratory-prepared goat anti-(human IgG) IgG-HRP (see Methods Sect 2.7, p 80 and Results Sect 1.1b, p 102), or commercially produced goat anti-(human, IgG, IgA, or IgM) IgG-HRP conjugate.

For assay of rabbit anti-sera, goat anti-(rabbit immunoglobulins) IgG-HRP was used.

"Carmarthen" antigen, Dried milk (Oster Milk Two, Farley Healthcare Products Ltd, Notts., U.K.) was prepared as follows. Dried milk (3g) was added to the coating buffer (20ml) which was vortexed then centrifuged (30,000g, 15min). The supernatant was diluted (1:100) in the same coating buffer and used to coat the microtitre wells (100µL/well) which were then incubated overnight at 4°C, or for 3h at 37°C.

(II) Xanthine Oxidase

The procedure was identical to that of the BMFGM ELISA above except that XO, prepared according to Methods Sect 2.4 (iii),p 76 , was used to coat the microtitre wells at a concentration of 15µg/ml of sodium carbonate buffer.

(III) Platelet membrane

The development of this ELISA from the method of Winiarski and Ekelund (1986), is discussed in Results Sect 6.1, p 184. Microtitre wells were coated with platelet membrane, prepared according to Method Sect 2.2 (ii). Platelet membrane was homogenised in 50mM-sodium carbonate buffer,pH 9.6 at a concentration of 10µg platelet membrane protein/ml carbonate buffer, the suspension (100µL) was added to each well and left to incubate at 4°C overnight or at 37°C for 3h. Wells were then washed (3 x 10 min) with PBS/Tween. Serum, or IgG (5mg/ml in PBS),

was diluted in Incubation buffer [PBS/Tween containing 4% (w/v) BSA, and 5% (v/v) Normal rabbit serum (NRS) v/v], at dilution factors of 1:100 and 1:200 respectively, and added to the wells (100µl/well). After incubation overnight at 4°C or for 2h at 37°C, wells were washed (3 x 10min) with PBS/Tween. Conjugate, diluted in Incubation buffer, was then added, (100µl/well), and left to incubate at room temperature for 2h. Wells were washed (3 x 10 min) and stained as for the BMFGM ELISA, (Methods Sect 3.3 (I). Conjugates used were approprate for the serum being assayed, (see BMFGM ELISA, Methods Sect 3.3 (I), p86).

4.0 TECHNIQUES

4.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was carried out as described by Laemmli (1970). Separating gel was prepared the day before the gel was run. The stacking gel used was of 5% polyacrylamide, and the separating gel of 10% polyacrylamide. Gel compositions are described in Table 12. Separating gel was poured between two glass plates and overlayed with water-saturated butanol, to allow polymerisation.After polymerisation, butanol was washed fom the surface of the gel with distilled water, the surface was blotted dry, and the stacking gel was then poured onto the separating gel.

Table 12 Chemical composition of SDS-PAGE gels.

Chemical	Separating (ml)	Stacking (ml)
Acrylamide stock (25% w/v, 24.5% Acrylamide, 0.62% Bis-acrylamide.	16	2
1M-Tris/HCl, pH8.8	15	-
1M-Tris/HCl, pH6.8	-	1.25
Distilled H_2O	7.7	6.3
The reagents overleaf were mixed, degassed, and the following reagents were then added:-		
10% SDS (w/v)	0.4	0.1
N,N,N',N'-Tetramethylethylene-diamine (TEMED)	0.025	0.01
1.5% Ammonium persulphate (w/v)	0.9	0.35

A well comb was inserted and again the surface of the gel was overlayed with water-saturated butanol. After polymerisation, (approximately 40min), the comb was removed and the gel was placed in an electrophoresis tank, (Protean II, Biorad Chemical Division Richmond, California, U.S.A.). Electrophoresis buffer was poured into the tank and the samples were loaded into the buffer filled wells with an Hamilton syringe. The gel was run at 70-80V (no greater than 30mA) until the dye front had reached the separating gel. The voltage was then increased to 150-200V, (again the current did not exceed 30mA), and allowed to run until the dye front had reached the bottom edge of the separating gel. The gel was then removed from the tank and treated accordingly.

Electrophoresis buffer contained, 0.192M-Glycine, 0.025M Tris, and 0.01% (w/v) SDS, final pH 8.3.

Generally, this buffer was made as a 10-fold concentrated stock. The buffer could be used to run 3 SDS-PAGE gels before disposal and approximately 2.5L were used in the tank.

Preparation of samples

Sample buffer contained 50% (w/v) glycerol, 10% (w/v) SDS, 10% (w/v) 2-mercaptoethanol, 0.01% (w/v) bromophenol blue, in 0.125M-Tris/HCl.

Samples, prepared in 1M-Tris/HCL, pH 6.8, were diluted in the above buffer (4:1 v/v), membranes being homogenised in sample buffer. Samples were then either incubated at 37°C for 2h, or boiled for 1min to ensure complete denaturation. It was also ensured that all

samples contained at least a 5-fold excess of SDS by weight over protein.

Molecular weight markers, SDS-6H (Sigma Chemical Co, Poole, U.K), were prepared as instructed using the above sample buffer.

50µl wells contained approximately 75µg protein for membrane samples, and 20µg for a single protein.

Staining of SDS-PAGE gels for protein

(i) Coomassie Brilliant Blue

Gels were fixed in 45% methanol/10% acetic acid in distilled water for 40 min, followed by staining with 0.1% Coomassie Brilliant Blue in the fixing solution for 1 h. Gels were then destained in 5% methanol/7.5% acetic acid in distilled water overnight,followed by fresh changes of destain until the gel background was transparent.

These processes were carried out at room temperature, with constant gentle agitation.

(ii) Silver Staining (for samples containing < 10µg protein).

The method used was that according to Morrisey (1981), which is a modification of the original method by Switzer et al (1979).

Slab gels were incubated succesively, for 30min each, in 50% methanol/10% acetic acid; 5% methanol/7% acetic acid and finally 10% glutaraldehyde. The gels were then washed overnight in a large volume of distilled water and incubated for 30 min in dithiothreitol (5µg/ml). Excess solution was removed and immediately, without

rinsing, silver nitrate (0.1% w/v) was added, and left to incubate for 30min.

The gel was then rinsed with developing solution [3% sodium carbonate (80ml) and 37% formaldehyde (20ml)] and soaked in developer until the desired level of staining was obtained. The reaction was stopped by the addition of 2.5M citric acid (5ml) directly to the developer, and mixing by agitation. The solution was discarded and the gel was washed 5 times with distilled water, then soaked in 0.3% sodium carbonate for 10 min. Finally, the gel was washed in distilled water and stored in the dark at 4°C.

4.2 Western-blotting of SDS-PAGE gels.

The method according to Towbin et al (1979) was followed.

SDS-PAGE slab gels were overlayed on nitrocellulose paper, (0.2µ, from Sartorius, Belmont, Surrey, U.K), pre-soaked in transferring buffer, ensuring that no air bubbles were present between the layers. The filter and gel were then sandwiched between blotting paper (Whatman 3M) and Scotch-brite pads (on both sides), before being clamped into a plastic holder.

The holder was inserted into a transfer tank, containing 2.5L of transfer buffer, 25mM Tris/HCl, 20% methanol, pH 8.3, ensuring that the nitrocellulose was adjacent to the anode and the slab gel was adjacent to the cathode. Protein was transferred from the gel to the nitrocellulose for 10h, at 60mA, and at 4°C.

The nitrocellulose filter was washed with distilled water, to remove any traces of SDS. Tracks were highlighted by immersion of the filter in 0.1% Ponceau S, in 3% TCA, when protein was stained as red bands (Coudrier et al, 1983). Tracks were cut with a sharp scalpel blade into 0.5cm widths and placed in incubation chambers. Residual Ponceau S was washed from the gels by incubation in PBS/Tween and the nitrocellulose strips, containing protein transferred from SDS-PAGE tracks, were then stained.

I Protein staining

The method according to Hancock and Tsang (1983) was followed. Indian ink (1µl/ml in PBS/Tween) was applied to each strip, ensuring complete immersion. When protein bands were visible, the ink was removed by aspiration, and the strip was incubated in PBS/Tween.

II Immuno-staining

Detection was according to the method of Towbin et al (1979, 1984), with some modifications.

Nitrocellulose strips were soaked, with gentle shaking, in blocking buffer (PBS/Tween, containing 0.1% (v/v) fish-skin gelatin) for 2h at room temperature. Strips were then treated with appropriate antibody i.e. human or rabbit serum (1:100), or IgG (1:200), diluted in incubation buffer (blocking buffer containing 5% Normal goat serum, and 4% BSA). Strips were incubated overnight at room temperature, then washed (4 x 15 min) with blocking buffer. A suitable conjugate was added see BMFGM ELISA Sect 3.3 (I),p87 , at 1:1000 dilution in

incubating buffer and left to shake at room temperature for 2h. Strips were washed as before, then stained as follows.

Stain for HRP conjugated IgG.

3-Amino-9-ethylcarbazole (AEC) [4% in dimethylformamide (DMF)] was diluted (1:20 v/v), with 50mM-sodium acetate buffer, pH5 to which 30% H_2O_2 (1µl/ml) was added. The inclusion of 0.05% Tween in this stain was found to prevent precipitation of AEC. Each strip was submerged in stain and was allowed to incubate, while shaking until, the strips showed brown/orange bands. The strips were washed in 50mM-acetate buffer, pH 5, then dried, and covered with film. Photographs were taken as soon as possible as the stain was found to fade with time.

Stain for Alkaline Phosphatase conjugated IgG.

To 10% diethanolamine buffer, pH9.8, containing 4mM-magnesium chloride (9vol), Nitroblue tetrazolium (1mg/ml in diethanolamine buffer (1vol) was added. In addition, 5-bromo-4-chloroindolyl phospate(5mg/ml in DMF) was added at 10µl/ml of stain. Nitrocellulose strips were submerged in stain, until blueish purple bands were visualised. The stain was then removed, and strips were immersed in distilled water and treated as above.

4.3 Immunoelectrophoresis

Barbitone buffer was prepared by adding barbital (5'-5' diethyl barbituric acid), 4g dissolved in distilled H_2O (150ml) heated to 95°C, to sodium barbital (5'-5' Diethylbarbituric acid, Sodium salt), 12g dissolved in distilled H_2o (800ml). The solution was made up to 1L,

and the pH adjusted to 8.2.

1.5% Agarose, (Electophoresis grade, Bethesda Research Laboratories, Gaithersburg, U.S.A) was made up in the barbitone buffer. Agarose gel (15ml) was melted, and poured onto a $10cm^2$ agarose gel support medium, (Gelbond, FMC corporation, Maine, USA).After gelling, wells were punched (3mm diameter), and troughs were marked between the wells. The wells were then filled with 5µl serum or IgG (1mg/ml). One well contained serum to which a few grains of Bromophenol Blue had been added. The flat bed electrophoresis tank was filled with barbitone buffer, and the gel was placed on a glass slide, connected to the buffer by a strip of filter paper, making contact with the complete edge of the gel and the buffer. A current of 20mA was applied until Bromophenol Blue had migrated to the edge of the gel.

The gel was then taken out and troughs were filled with an appropriate antiserum and left to incubate in a humid chamber until precipitin lines appeared. The gel was rinsed succesively in PBS and H_2O for 24h each, then stained by immersion in 0.1% (w/v) Ponceau S in 3% (w/v) TCA. The gel was then dried to a film by incubation at 37°C for 24h.

4.4 Platelet aggregation

Preparation of Platelet Rich Plasma (PRP)

Fresh blood was collected in heparinised vacutainers, from hospitalised patients at the West Wales Hospital (Carmarthen, Wales, U.K.). Blood group "O" samples were

detected as follows. One drop of a murine monoclonal anti-(human A and B blood group) antibody (Orthodiagnostics, New Jersey, U.S.A) was added to a sample of blood (approximately 1ml). The samples were shaken gently, then centrifuged (160g,2min). Samples which showed agglutination were discarded. The remaining blood group "O" samples were centrifuged (160g,10min) and the supernatant was collected as PRP. If any red blood cell contamination was present, the solution was recentrifuged for another few minutes. Platelet Poor plama was prepared by re- centrifugation of the remaining blood specimen (30,000g,10min).

Platelet aggregation

Platelet aggregometry was performed using a whole blood aggregometer from Coulter Electronics Ltd. (Luton, Beds. U.K.). Platelet aggregation denotes the adherance of platelets to each other. The phenomenon can be induced by adding aggregating agents to PRP or washed platelet suspensions. The process of aggregation is measured spectro-photometrically, using light transmittance through PPP, as a measure of 100% aggregation. The instrument develops a voltage proportional to the transmittance of light through PRP, and the voltage is recorded on a strip recorder as a function of time.

Aggregation of PRP

Each batch of PRP, prepared from fresh platelets as described above, was first tested for its ability to aggregate with collagen, a known aggregant. PRP (0.5ml)

of platelet count 2-7 x 10^{12}/L, was added to the aggregation chamber and left to reach 37°C. Collagen (2-4 µg/ml) was added, the solution stirred by means of a magnetic stirrer, and aggregation was observed. If the PRP did aggregate, then the test aggregant, in this case pre-, and post-immune sera, would be added (50-100µL aliquots),to a fresh aliquot of the same batch of PRP. Every 30 min, PRP would be re-tested with collagen, and it was noticed that generally, freshly prepared PRP was viable for up to 3h.

Aggregation of washed platelets

In the same way as PRP, washed platelets (10-50 x 10^{10}/L), prepared according to Methods Sect 2.3,p74, were placed in the aggregating chamber and tested for their ability to aggregate with collagen. Another sample of the same washed platelet suspension was then treated with test aggregant i.e. serum, or IgG (5mg/ml), in 50-100µL aliquots.

Aggregation was usually observed within 5 min.

5.0 STATSITICAL ANALYSES.

5.1 Kolmogorov - Smirnov test

This method of analysis was used when comparing antibody levels in two populations (e.g. control and MI sera).

The Kolmogorov-Smirnov two tailed test (Siegel,1956) considers the difference between cumulative frequencies of two samples of populations. The test identifies the part of the distribution, in which maximum difference

occurs, and a value (Ks) is directly derived from it. This is compared with a theoretical value Ks, dependant upon the numbers of each sample, and the required level of significance. If the observed Ks is less then the theoretical value of Ks, then the null hypothesis holds true, that the two samples have been drawn from the same population, and are not significantly different. Conversely, if the observed Ks is greater than or equal to the theoretical Ks, then the two samples are sufficiently different to suggest that they are from two different poulations.

5.2 Chi² "Goodness of fit" test.

This test was used when comparing frequencies of antibody distributions within different categories, such as age group.

This analysis (Parker,1973; Hayslett,1976; Robson,1979) is used to test the significance of deviations between observed (O), and (E), expected frequencies. The chi² statistic computed is a goodness of fit between observations and theoretical values.

The test of association between frequency distributions in different categories is considered by looking at each individual frequency within a particular category as a cell in a Table of columns (i.e.categories), and rows (i.e. frequencies). Chi² is calculated in the form:-

$$\frac{(O-E)^2}{E}$$

for each cell, then summed, and is compared with tabulated values, dependent upon the degrees of freedom for each analysis.

5.3 Rank correlation

This test was used, for example, to compare results from two assays carried out on the same set of samples. If a random sample of n members and two characteristics, x and y of each member are measured, a random sample of n pairs of observations is obtained. Under the assumption that the distribution of x and y are normal, the sample correlation co-efficient is a measure of the linear relation between the two variables. If, however, the values of x and y are not normally distributed, then the rank-correlation co-efficient can be calculated, which depends upon the ranks of values, rather than actual measurements. Values in the sample are ranked from 1 to n, and for samples of the same value, then their average rank is allocated to each. The Spearman rank-correlation co-efficient, Rs, is calculated as follows:-

$$Rs = 1 - \frac{6 \ (Xi - Yi)^2}{n(n^2 - 1)}$$

Where n = Number of pairs of observations.

Xi = rank of xi

Yi = rank of yi

A value of Rs = 1, indicates perfect agreement between the ranks: a value of -1 indicates that the ranks of x are exactly opposite to those of y,(Parker, 1973).

RESULTS

1.0 DEVELOPMENT OF AN ELISA TO DETECT ANTI-BMFGM ANTIBODIES.

1.1 Design of a BMFGM ELISA

The design of the above ELISA system was based upon the following protocol used in most ELISA systems. Microtitre plate wells were coated with antigen (i.e. BMFGM, Methods Sect 2.1, p70) suspended in 50mM-sodium carbonate buffer, pH 9.6, and incubated overnight at 4°C or for 2h at 37°C. Wells were washed, (3 x 10 min), with washing buffer [0.05% Tween 20 in PBS, 200µl/well] and incubated with human serum, at an appropriate dilution in washing buffer, overnight at 4°C or for 2h at 37°C. Wells were then washed as above and incubated with goat anti-(human IgG)-HRP conjugate (Materials and Methods Section 2.7,p80), at an appropriate dilution, in washing buffer for 2h at room temperature, before washing as before. Finally, enzyme substrate (420µM-tetramethylbenzidine in 0.01M sodium acetate/citrate buffer,pH6, containing 0.3mM H_2O_2) was added and allowed to incubate until a blue colour developed, when the reaction was stopped by the addition of 2M H_2SO_4 (50µl/well) and absorbance was read at 450nm. All additions were at 100µl/well unless otherwise stated.
The following parameters were investigated to optimise this ELISA:-

a. Coating conditions

ELISA plates were coated with various concentrations of BMFGM, to find the optimal coating concentration for

the assay. Pooled high titre (anti-BMFGM antibodies) human serum, diluted 1/200, was assayed using laboratory prepared goat anti-(human IgG)-HRP conjugate (1:1000). Fig 25, p103, illustrates that maximum absorbance was reached between 8-12 µg BMFGM protein /ml coating buffer. A coating concentration of 10 µg BMFGM protein /ml was used routinely in the BMFGM ELISA.

b. Conjugate concentration

Horseradish Peroxidase (HRP) conjugated to goat anti-(human IgG), prepared in the laboratory (see Methods Sect 2.7, p80), was titrated at various concentrations on ELISA plates, coated with 10µg BMFGM protein/ml, and incubated with a pooled high titre (anti-BMFGM antibodies) human serum, diluted 1/100. A conjugate concentration of 1µl/ml provided a sufficient colouration, after a period of incubation of 10 min (Fig 26, p103). Any subsequent conjugate preparation was compared directly to previous batches, to maintain consistency.

c. Serum dilution

Plates were coated at 10µg BMFGM protein/ml, and human sera at dilutions ranging from 1/25 to 1/800 were assayed using laboratry prepared conjugate, diluted 1/1000.

Because so many different sera had to be assayed, only one dilution of each serum was made; a 1/200 dilution was used. This was the minimum dilution necessary to give consistent titres, particularly for higher titre sera (see Table 13, p104).

Fig 25 Effect of varying BMFGM coating concentration on the ELISA.

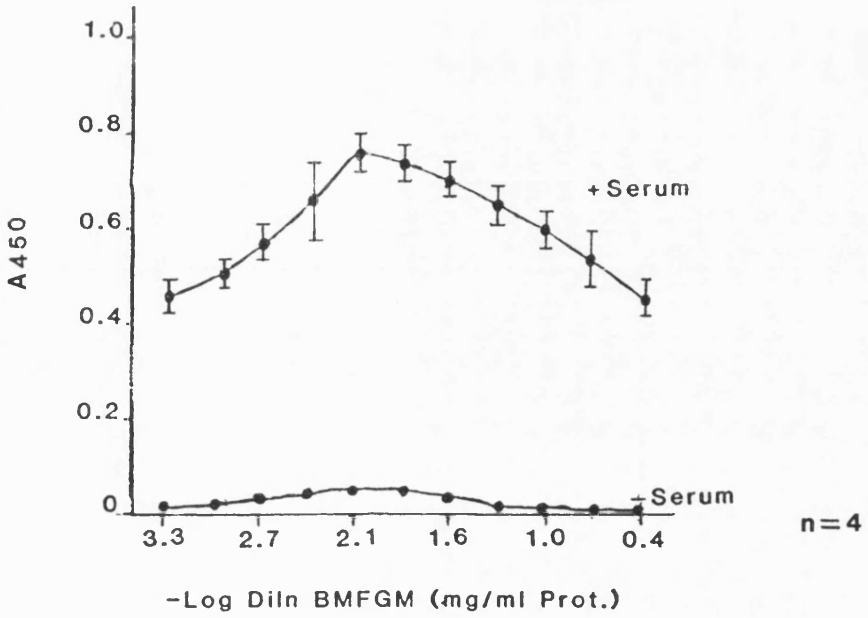
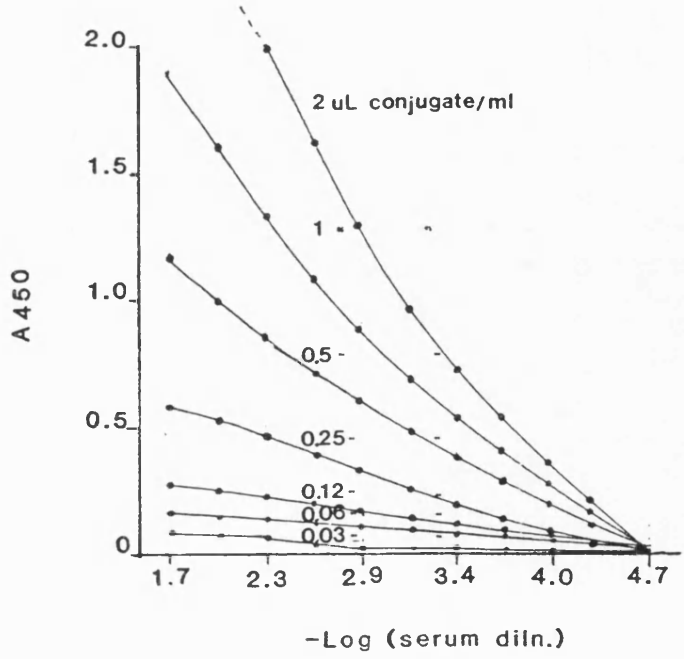


Fig 26 Effect of varying conjugate concentration on the BMFGM ELISA.



The absorbance values, quoted above, represent the mean of 8 readings obtained on a particular day.

Table 13 Effect of serum dilution on resultant antibody titre.

Serum No	Serum dilution					
	1/25	1/50	1/100	1/200	1/400	1/800
1	5.3	5.1	4.5	4.7	4.7	4.5
2	5.9	6.0	5.2	5.0	3.7	6.5
3	6.3	6.7	9.3	10.0	11.2	10.8
4	8.9	17.0	19.9	20.0	21.4	21.0
5	16.4	17.7	19.0	21.6	22.4	22.6
6	37.0	22.3	20.9	21.5	23.4	22.0
7	42.1	65.9	53.7	51.4	49.2	50.6
8	64.6	52.4	56.2	59.0	55.2	48.2
9	93.3	69.0	48.7	61.8	64.6	63.5
10	104.0	99.8	58.9	79.6	78.0	71.3
11	64.2	81.4	70.8	120.5	132.5	142.3
12	89.4	120.6	150.7	168.6	173.1	169.4

Titre is expressed as a percentage of the standard.

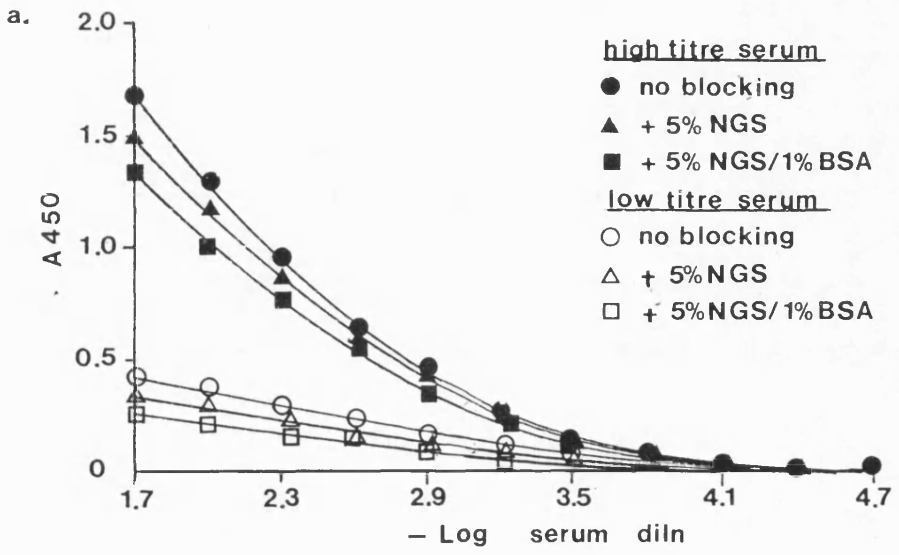
d. Blocking conditions

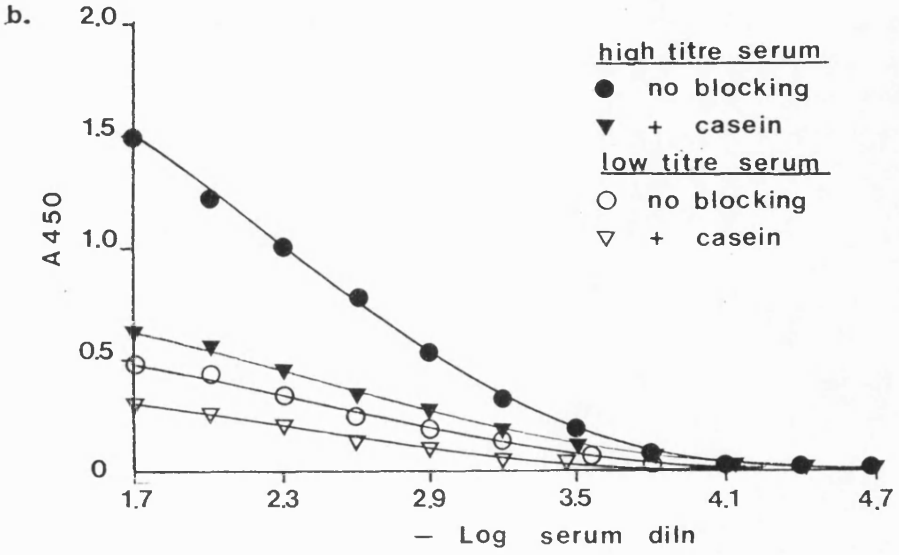
Various blocking conditions are commonly used to minimise non-specific binding (Ruttenberg et al,1976; Lin and Kasamatsu,1983;Johnson et al,1984), which can occur in either of two ways. The first is that conjugate may bind to the antigen coating the plate, thus an absorbance would be detected with no sera present: the second is that antibodies, which are not specific for antigen, may bind, thus the conjugate would detect both specific and non-specific antibody binding.

Conditions were tested using a coating concentration of BMFGM at 10µg/ml, pooled human serum was diluted at 1/200, and laboratory prepared conjugate was diluted 1/1000. Fig 27a, p106, illustrates that including 5% normal goat serum (NGS) in the washing buffer (with or without 1% BSA), reduces the absorbances of both the high and low titre sera. However, as both high and low titre sera were equally affected, it did not seem crucial to include either of these blocking agents in the BMFGM ELISA, as the relative differences between antibody titres would still be the same. This was also obvious from other sera tested (results not shown).

The effect of casein (1% in the washing buffer, and 0.1% during incubation of both sera and conjugate), assayed using the same conditions as for the blocking agents above, was to diminish the absorbance produced in all sera assayed (e.g. see fig 27b, p106), thus it was not included in the assay. When PBS containing 0.05% Tween was used as a blocking and incubating

Fig 27 Effect of using a) NGS (5%) ± BSA (1%) and b) Casein (1%) as additional blocking agents in the BMFGM ELISA.





buffer, blank wells i.e. with buffer alone, had zero absorbance. Various sera gave rise to a range of absorbances, from values greater than 2 (high titre sera) to absorbances comparable with those of the blank wells (low titre sera). Blocking with PBS/Tween alone was accordingly used in the BMFGM ELISA.
The finalised protocol for the BMFGM ELISA is described in Materials and Methods Sect 3.3, p 86.

1.2 Nature of binding of anti-BMFGM antibodies to the membrane

To examine whether the binding of anti-BMFGM antibodies to the membrane was specific or non-specific, F(ab')2, Fab', and Fc fragments were prepared from two high and two low titre sera.The fragments were assayed according to the ELISA system (Methods Sect 3.3 (i), p86),using a 1:50 dilution of a 1mg/ml solution of each,in place of sera. For high titre sera, binding of IgG, F(ab')2, and Fab' fragments gave high absorbances (Figs 28a, 28b and 28c, p109), whereas that of Fc did not (Fig 28d). Low titre sera IgG, $F(ab')_2$, and Fab' fragments gave low absorbances, which were nevertheless higher than these produced by high titre sera Fc fragments. Low titre sera Fc fragments produced similar absorbances to those of high titre Fc fragments.

For 50 samples, anti-BMFGM antibody levels were compared to total IgG levels (measured at BATH RUH), in the same serum sample. Fig 29, p110, shows the results of this

Fig 28 Diagrams showing titration of :

a) IgG,

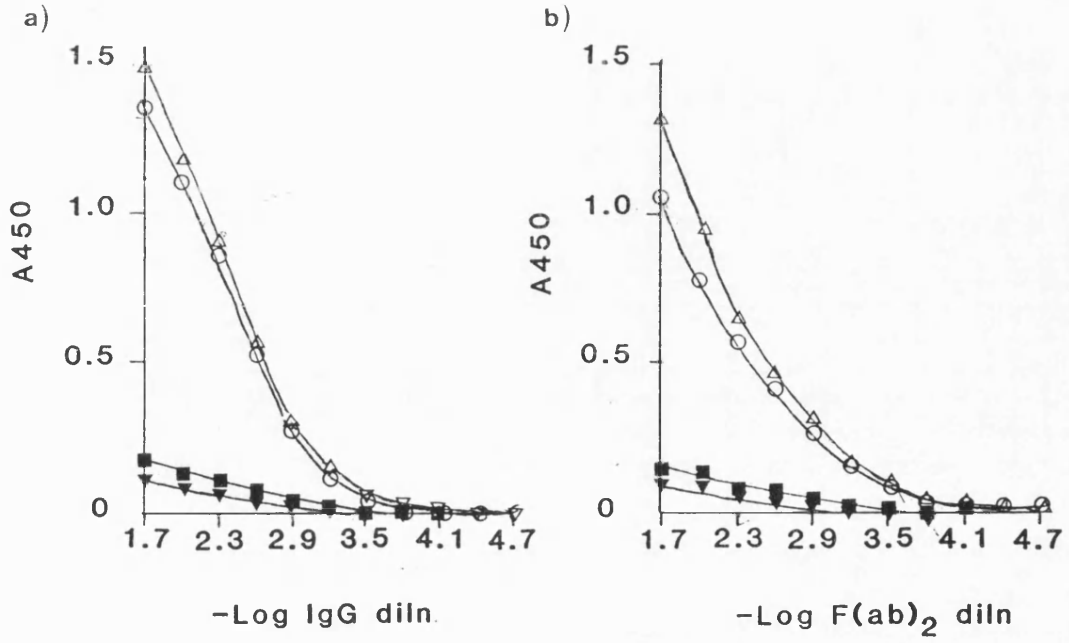
b) $F(ab')_2$,

c) Fab' and

d) Fc fragments, prepared from high and low titre anti-BMFGM serum.

Key:-

△ -	High titre serum	100% Std
○ -	"	75% Std
■ -	Low "	5% Std
▼ -	"	2% Std


a)
b)
1.5
1.0
0.5
0
A450
1.7 2.3 2.9 3.5 4.1 4.7
-Log IgG diln
-Log F(ab)2 diln

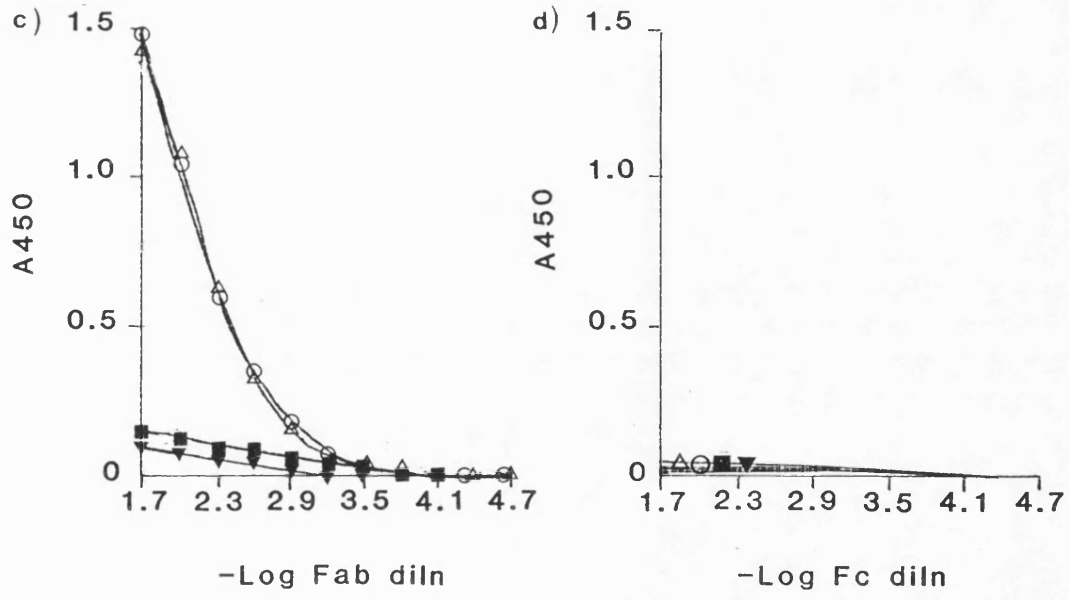
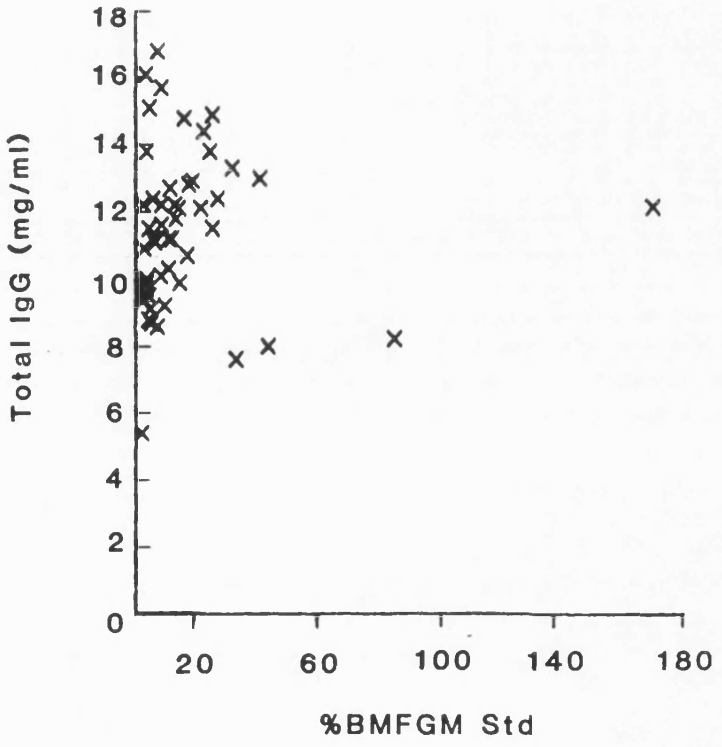
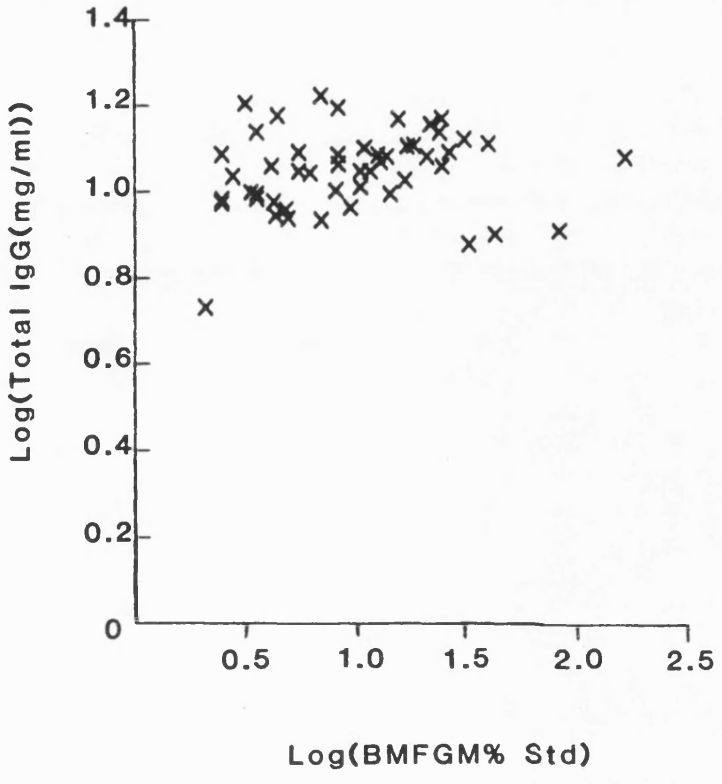

c)
d)
1.5
1.0
0.5
0
A450
1.7 2.3 2.9 3.5 4.1 4.7
-Log Fab diln
-Log Fc diln

Fig 29 Graph showing a comparison of total IgG versus anti-BMFGM antibodies.





comparison. The rank correlation coefficient (Rs) calculated for these data is 0.220. For p=0.05, only values of 0.273 or above show a significant correlation i.e. the results indicate that anti-BMFGM antibody levels are independent of total IgG levels.

1.3 Routine assay of samples

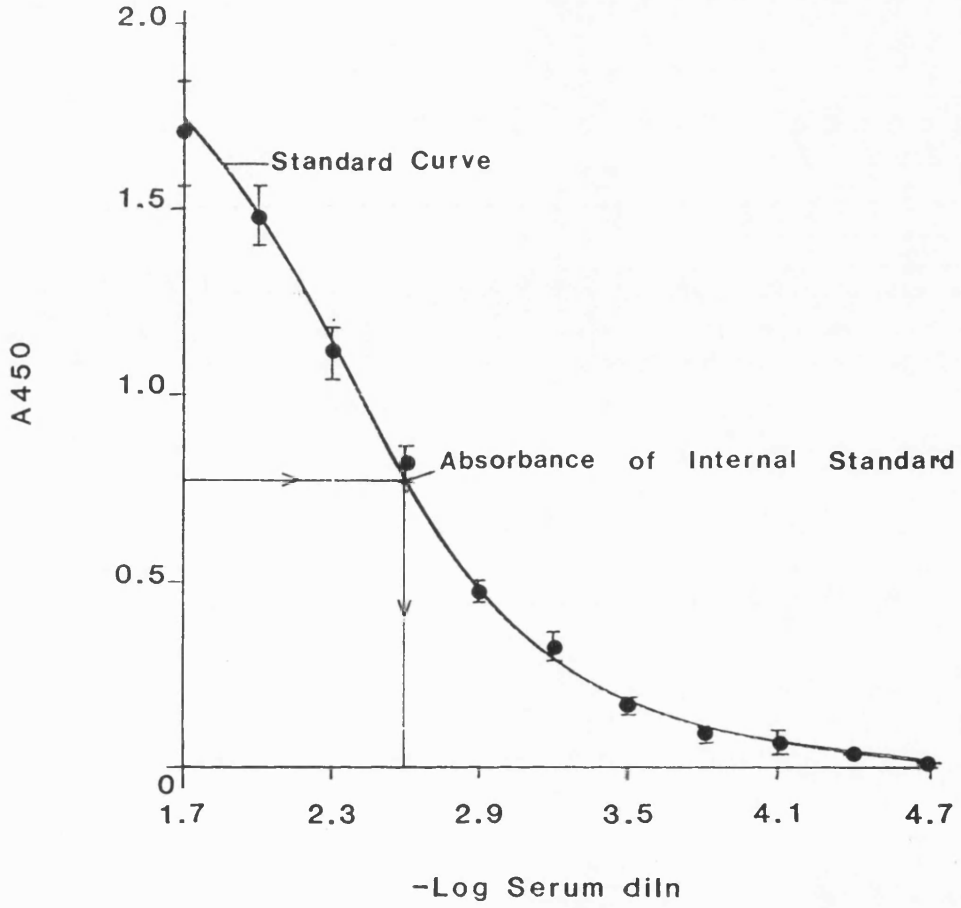
An important requirement of the ELISA is that the results be directly compared from day to day. To ensure this:-

1. A number of high titre sera were pooled and samples were used to derive a standard curve for each assay plate. Titres of test sera could then be expressed as a % of the standard serum (% Std).
2. A second pooled serum was prepared and assayed on every plate. If its titre deviated greatly from the mean, the samples contained on that plate would be re-assayed (see Fig 30, p112).

Each test sample was assayed in triplicate and the mean value was quoted. If the range of triplicate absorbances varied by more than 0.05 units that sample would be re-assayed.

Table 14, p113, shows the results of titrations of 5 different sera, assayed on 10 separate occasions and the variation to be obtained within each of these ranges.

Fig 30 Diagram showing the standard curve used in routine ELISA's, prepared from pooled sera, and the value expected of the internal standard.



Absorbance values of the Standard Curve are ± Standard Error (n = 4).

Internal Standard = 50.2 ± 4.6 %Std (n = 10).

Table 14 Variations present in anti-BMFGM titres

		Serum number				
		1	2	3	4	5
Assay number	1	5.3	11.4	19.2	70.4	139.8
	2	5.2	10.8	21.6	100.2	120.4
	3	5.1	13.2	25.4	86.4	160.2
	4	6.0	10.2	22.4	96.3	125.6
	5	3.6	11.0	21.3	90.2	186.4
	6	4.5	11.6	18.4	86.4	140.4
	7	4.6	12.1	19.8	86.1	140.3
	8	4.9	10.5	22.0	70.8	160.9
	9	2.3	13.4	21.6	100.0	133.6
	10	3.4	9.6	21.6	69.2	154.2
Mean		4.5	11.4	21.3	85.6	146.2
S.D.		1.0	1.2	1.8	11.3	18.6
Range		2.3-6.0	9.6-13.4	18.4-25.4	69.2-100.2	120.4-186.4

Titres are expressed as a % of BMFGM Std.

2.0 ASSAY OF MRC MI AND CONTROL SERUM SAMPLES.

1.Serum samples were assayed from three sets of male MI patients:-

a). 2-5 weeks post infarct (termed new)

b). 6 months post infarct

c). 2 years ,, ,,

and from control patients (for further details see Materials Section 1.2, p69).

2.1 Comparison of "New" MI with control samples

Fig 31, p115, shows the results of a comparison of New MI samples (< 65yrs of age), with control samples. There is no obvious difference between the two populations. A Kolmogorov-Smirnov (K-S) analysis shows that the greatest difference between the two populations occurs above 10-20% of the Std (i.e. 28.6% of MI, and 32.7% control samples have values greater than this). However, this difference is not significant.

2.2 Comparisons of "New", 6-month, and 2-year samples.

Fig 32, p116, illustrates anti-BMFGM antibody levels in each of the categories of MI patients listed in 2.0 above. There appears to be little difference in antibody distribution between new, 6-month, or 2 year patients. A chi^2 analysis of the different MI categories with Ab ranges of 0-10,10-20,20-30,30-40,40-50, and 50+% of Std give a calculated value of 13.694 (18.307 is necessary for significance at p=0.05), showing that anti-BMFGM

Fig 31 Distribution of anti-BMFGM antibodies in 462 "New" MI samples (<65yrs of age), with 517 control samples.

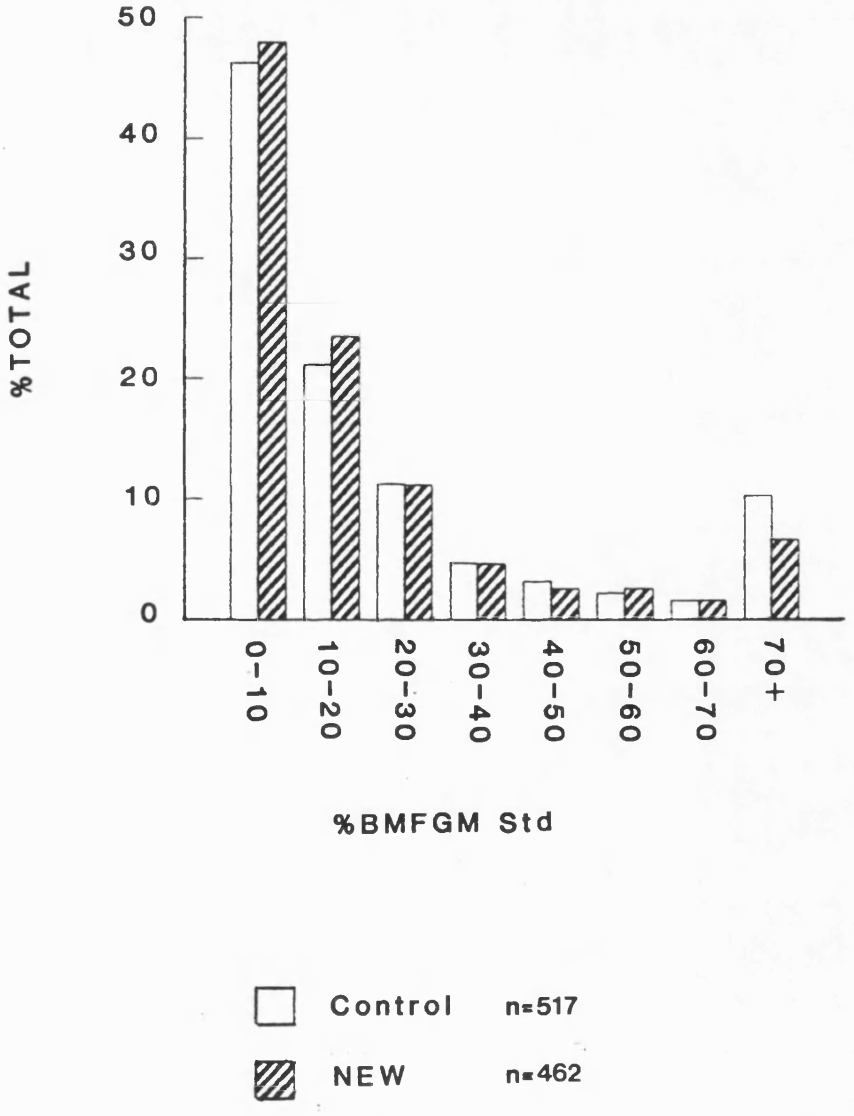
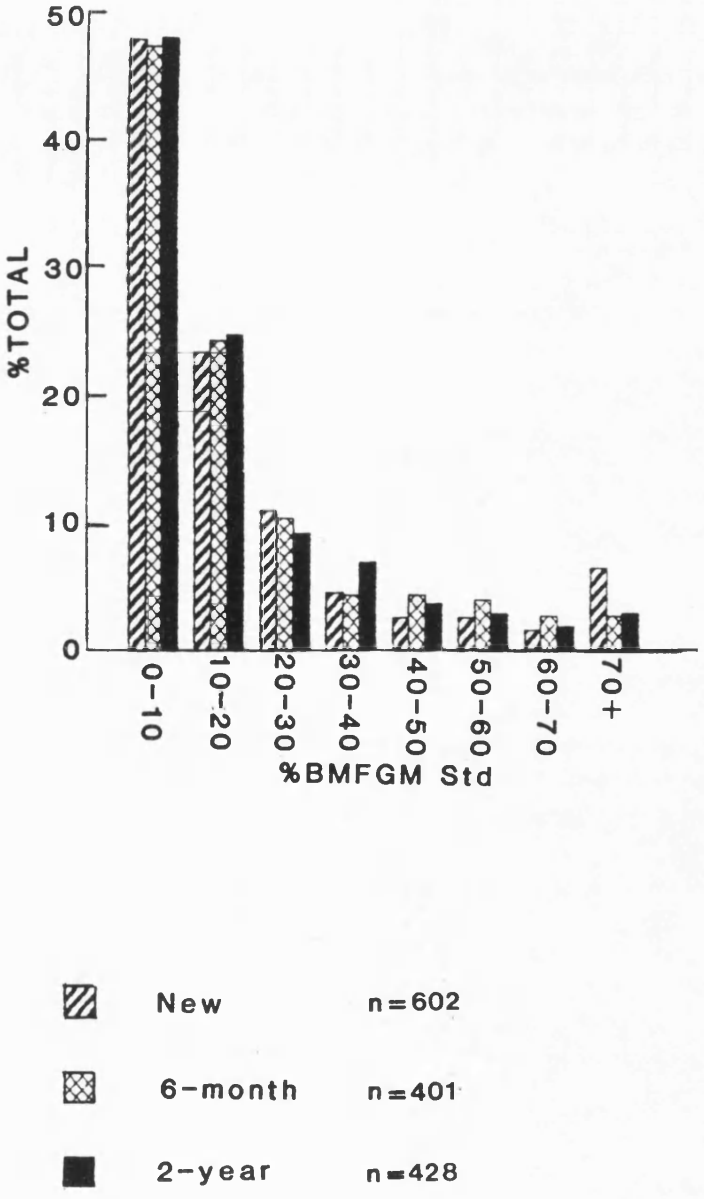


Fig 32 Distribution of anti-BMFGM antibodies in:-
NEW, 6-MONTH, 2-YEAR, MI patients



antibodies are similarly distributed within each of the three groups.

Investigation of changes in anti-BMFGM titres (new - 6-months, and 6-months - 2-years) post infarct.

Patient antibody levels were compared directly in pairs of time intervals after infarct. Figs 33a and b, p118, show that there is a significant correlation between both new and 6-month antibody levels, and 6-month and 2-year antibody levels, suggesting that the anti-BMFGM titre after infarction is constant for up to 2 years (For new and 6-month samples Rs calculated = 0.947, values above 0.3 are necessary for a significant correlation at p = 0.05 and for 6-month and 2-year antibody levels Rs calculated = 0.957, values above 0.352 are necessary for a significant correlation at p = 0.05)

2.3 Comparisons of "new", 6-month, and 2-year MI with control samples (considering only titres of less than 10% of Std).

Approximately 50% of all of the groups of samples assayed have a titre of below 10% of the standard, and it is possible that any difference present between the groups may be present in this range. Fig 34a, p119, shows the results of a comparison between control samples and new MI samples. A K-S analysis shows that the two populations differ most above 3.0-4.0% of the Std (i.e. 67.9% of MI and 70.2% of control samples

Fig 33 A comparison of anti-BMFGM antibody levels in MI patients:-

a) First sample after infarct, with 6-month sample after infarct. **(229 samples)**

b) 6-month sample after infarct, with 2- year sample after infarct. **(31 samples)**

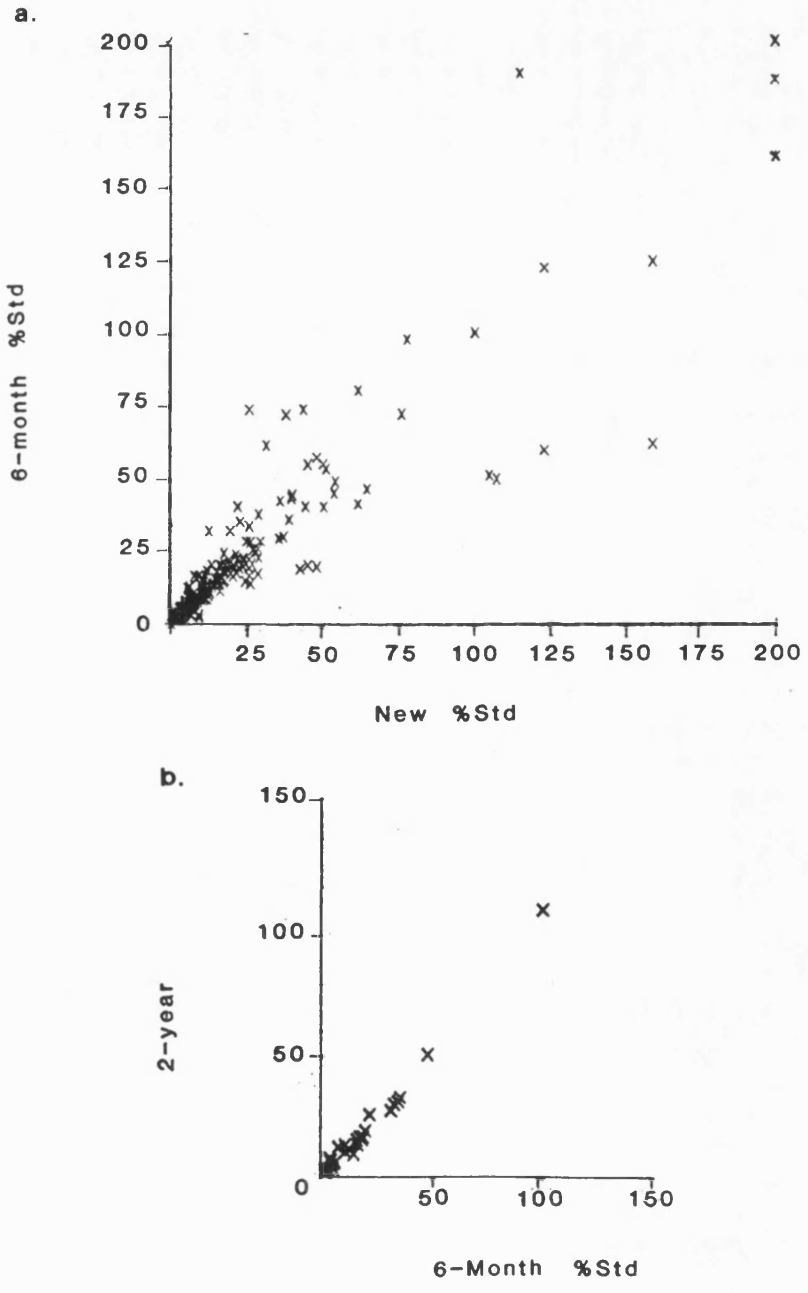
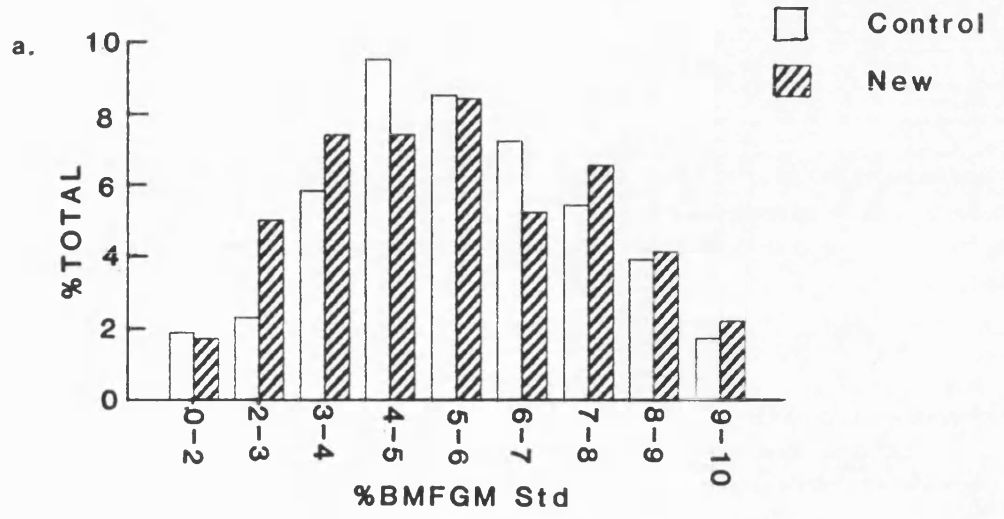
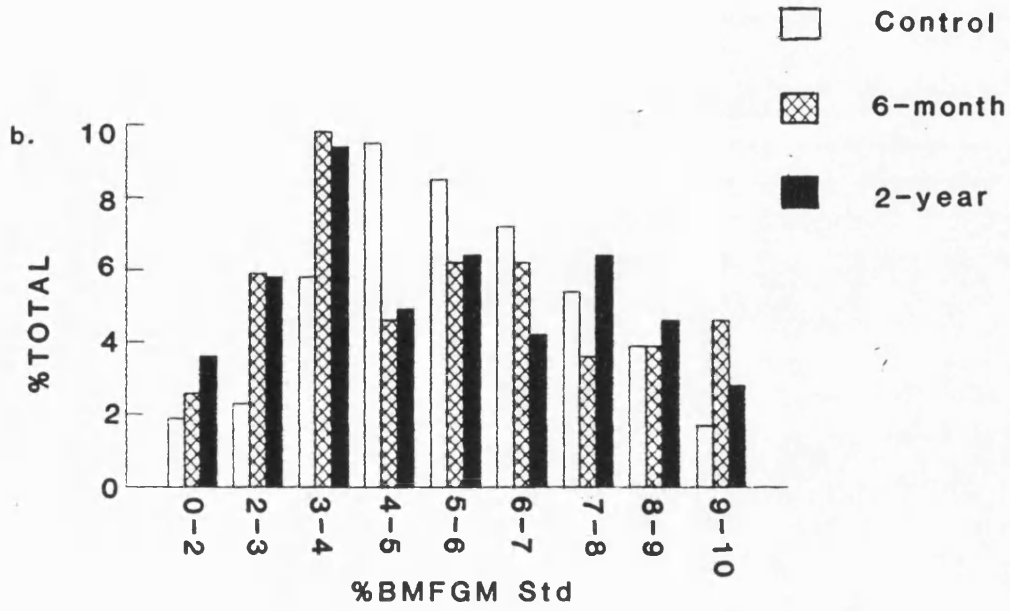


Fig34 A comparison of anti-BMFGM antibody levels of less than 10% of the Std in:-

a) New MI patients with control

b) 6-month and 2-year MI patients with control





considered have values greater than 3.9% of the Std). This difference is not significant. Fig 34b, shows a similar comparison between control samples, 6-month and 2-year MI samples. Here, using the same analysis, a significant difference was obtained between both 6-month and 2 year samples and controls. A K-S analysis showed a maximum difference between both sets of samples to occur above 3.0-4.0% of Std (i.e. 59.3% of 6-month and 78.1% of control samples had titres of greater than 4.0% of the Std, as did 59.5% of 2-year and 78.2% of control samples. The difference however demonstrates a higher level of anti-BMFGM antibodies in the control samples.

2.4 Regional variation in anti-BMFGM titres of new MI samples

MI samples were collected from various regions of Wales and South-West England. To examine whether there was any regional variation, titres obtained from "new" samples were examined according to their area of origin. These are compared in Table 15a, p122, and 15b which also compares the results when patients of >65 years of age are excluded.

A chi^2 goodness of fit analysis of all regions against antibody levels (in the ranges 0-10, 10-20, 20-30, 30-40, and 40+ (%Std)) showed that antibody frequencies are not significantly different in any region. Chi^2 values were calculated to be 7.648 (all samples) and 4.593 (<65 yrs of age). A value of 26.3 ($p=0.05$), is the minimum value necessary to show a significant difference.

Table 15a The regional distribution of anti-BMFGM antibodies (expressed as a percentage of the regional total (n)), considering all samples within the region and those of less than 65 yrs of age.

Key:-

A= all samples.

B= samples < 65yrs of age.

AREA 1 Cardiff and Newport

" 2 Swansea

" 3 South Wales Valleys

" 4 Bristol and Bath

" 5 Gloucester

Table 15b A comparison of the weighted mean for all regional new MI samples and those below 65 yrs of age with control samples.

REGION	1		2		3		4		5	
%Std	A	B	A	B	A	B	A	B	A	B
0-10	46.0	47.3	46.3	46.6	51.1	50.3	53.2	52.8	48.3	50.0
10-20	23.6	25.0	23.2	24.1	22.3	24.8	14.9	16.7	24.1	15.0
20-30	10.8	10.6	11.6	13.8	13.3	10.6	10.6	11.1	13.8	8.3
30-40	4.4	4.3	4.3	5.2	3.2	3.5	6.4	5.6	0.0	0.0
40-50	3.6	2.7	2.9	0.0	2.7	2.8	0.0	0.0	3.4	4.2
50-60	1.6	1.6	2.9	1.7	1.1	1.4	2.1	2.8	3.4	4.2
60-70	3.2	2.1	0.0	0.0	1.1	0.7	2.1	2.8	3.4	4.2
70+	6.8	6.4	8.7	8.6	5.3	5.7	10.6	8.3	3.4	4.2
n=	250	188	69	58	188	141	47	36	29	24

	WEIGHTED MEANS		CONTROL
	A	B	B
0-10	48.4	48.7	46.2
10-20	22.5	24.1	21.1
20-30	11.8	10.9	11.2
30-40	3.9	4.0	4.6
40-50	2.9	2.2	3.1
50-60	1.7	1.8	2.1
60-70	2.1	1.6	1.5
70+	6.7	6.5	9.9
n=	583	447	517

All titres are expressed as % of BMFGM Std.

Table 15b, p122, shows that when the weighted means of titre categories within each of the regions (with and without considering samples of >65 years of age) are compared with each other and with the control group there are in fact very similar in distributions.

2.5 Distribution of anti-BMFGM antibodies within age groups of New MI samples.

Table 16, p124, shows a comparison of anti-BMFGM antibody levels within age groups. Although a statistical analysis shows no relation between age group and antibody levels it is worth noting that the age group containing the largest deviations from the expected results are in the 56-60 year age group. A chi^2 analysis comparing the age groups (as shown in Table 16), with anti-BMFGM antibody titres (in the range; 0-5, 5-10, 10-15, 15-20, 20-30, and 30+ % Std) gave a calculated value of 22.158; values of above 37.653 (p=0.05),are necessary to show that there is an association.

2.6 Comparisons of anti-BMFGM titres in other disease states.

Sera in the department were available from disease states in which there is an immunological disturbance; Myasthenia Gravis (MG) (Vincent,1979;Harrison and Behan 1986), Motor Neurone Disease (MND) (Digby et al,1985;Rutter et al,1986) and Idiopathic Thrombocytopenic Purpura (ITP) (Kelton and Gibbons, 1982; MacDonald and Savoca,1985). Anti-BMFGM levels of antibodies were assayed in each of these, and were compared

Table 16 Distribution of anti-BMFGM antibody levels within age groups.

Ab Titre	Age Group (years)					
(% Std)	<45	46-50	51-55	56-60	61-64	65+
0-5	21.2	20.9	15.5	16.7	22.8	19.1
5-10	25.6	27.6	27.8	32.5	26.0	26.1
10-15	15.2	13.6	13.4	20.0	8.7	14.8
15-20	4.5	15.5	8.2	10.0	11.8	8.7
20-30	13.6	12.1	16.5	6.7	11.8	11.3
30-40	7.6	3.4	6.2	2.5	5.5	4.3
40-50	1.5	3.4	4.1	0.0	3.9	3.5
50-60	1.5	0.0	1.0	5.0	2.4	0.9
60-70	1.5	0.0	2.1	1.7	0.8	2.6
70+	7.6	3.5	5.2	5.0	6.3	8.7
n=	66	58	97	120	127	115

Frequencies are expressed as a % of total number of samples within each age-group.

to the same controls as used in the comparisons with MI samples (Figs 35a, b and c, p127). MND samples showed no deviation from control subjects. However, although ITP and MG sera both appeared to show elevated levels of anti-BMFGM antibodies, the Kolmogorov-Smirnov analysis showed that there was only a significant difference in the MG sera compared to controls.

[A K-S analysis comparing MND sera with controls showed that the region of greatest difference occurred above 5-10% of the Std,(56.8% of MND, and 47.5% of control samples had titres of greater than 9.9% of the Std). However the calculated K-S value of 9.3 was less than the theoretical value of 30.4 (p=0.05) necessary to show a significant difference. Comparing ITP sera with control sera ,again the point of greatest differnce between the two sets of samples occurred above 5-10% of the Std (77.5%, and 50% of ITP and control sera, respectively, had anti-BMFGM titres greater than this). A calculated K-S value of 27.5 was less than the necessary value of 30.4 as above.

Comparing MG sera with control sera, the region of maximum difference was above 10-15% of the Std (82% of MG samples and 37.1% of controls had values greater than this). A calculated K-S value of 44.9 was greater than the necessary value of 30.4, showing that anti-BMFGM titres were significantly raised in MG sera compared to controls (see Fig 35c,p 127)].

Milk and dairy products have often been implicated as migraine triggers (Egger et al,1983; Monro et al,1984)

Fig 35 A comparison of anti-BMFGM antibody titres in control samples with a) MND
b) ITP
c) MG sera.

Key to anti-BMFGM antibody titre ranges:-

Range No	% BMFGM Std
1	0-5
2	5-10
3	10-15
4	15-20
5	20-30
6	30-40
7	40-50
8	50-60
9	60-70
10	70+

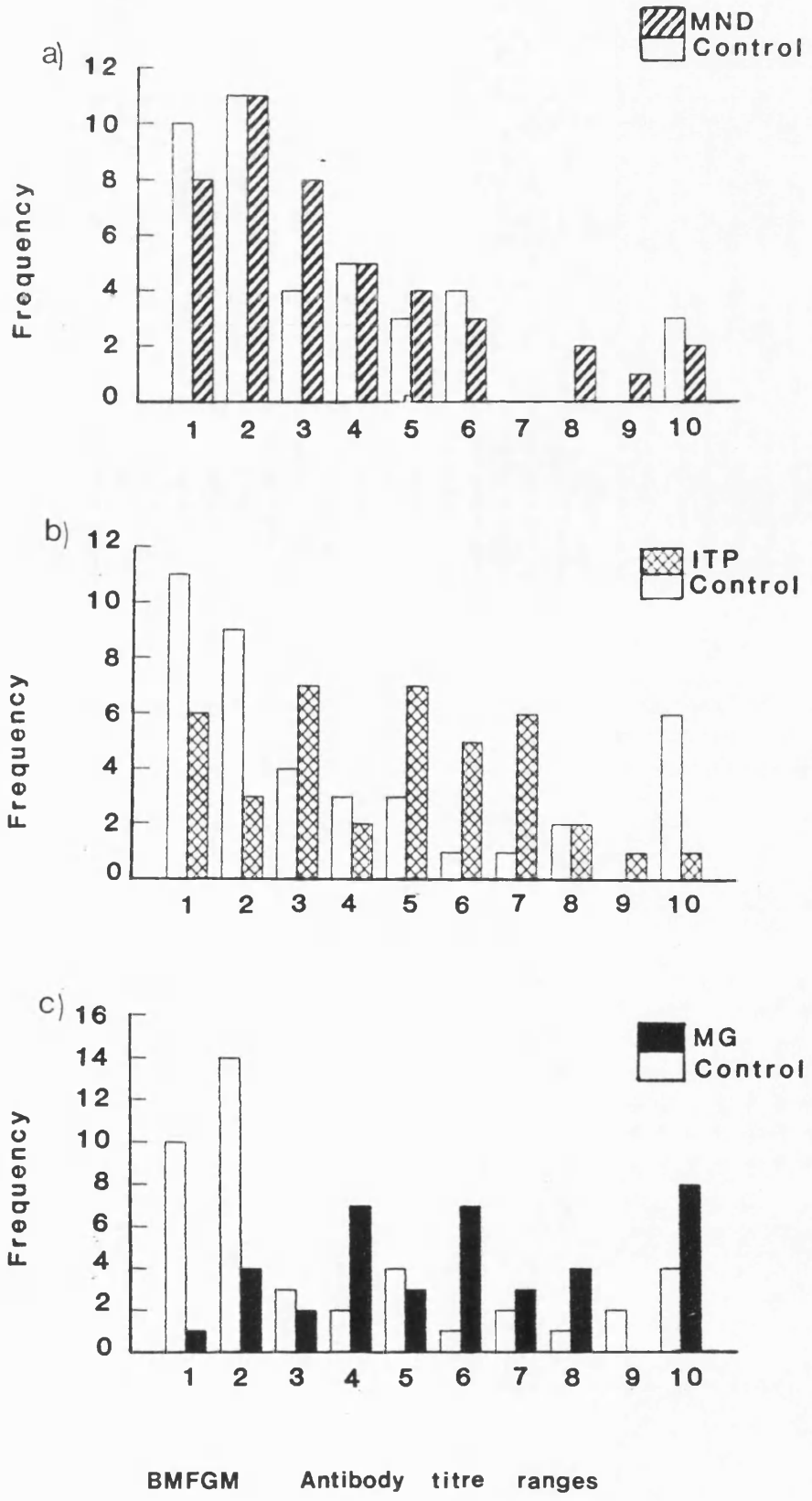

a)
MND
Control
Frequency
0
2
4
6
8
10
12
1 2 3 4 5 6 7 8 9 10
b)
ITP
Control
Frequency
0
2
4
6
8
10
12
1 2 3 4 5 6 7 8 9 10
c)
MG
Control
Frequency
0
2
4
6
8
10
12
14
16
1 2 3 4 5 6 7 8 9 10
BMFGM Antibody titre ranges

Table 17 Comparison of anti-BMFGM antibody levels with migraine trigger sources.

Ab Titre % BMFGM StD	Migraine trigger	Sex
0.0	Stress	M
0.0	Chocolate	F
0.0	Milk	F
0.0	?	F
0.0	Stress	F
0.9	?	F
2.9	Stress	F
5.6	Dairy Products	M
7.1	Menstrual	F
7.1	?	F
11.0	Stress	F
11.7	Stress	F
14.9	Dairy Products	F
21.9	Dairy Products	M
33.6	?	F
48.6	?	F
50.2	?	F
58.5	?	F
83.3	Chocolate	F
85.5	Menstrual	F
109.2	Stress	F
127.9	?	F
141.2	?	F

? = Unknown trigger

M = Male

F = Female

and it was considered worthwhile to investigate the levels of anti-BMFGM antibodies in the sera of migraine sufferers.

Sera were assayed blind and the results were then compared to the migraine trigger source (if known), see Table 17, p128. Of the known triggers those associated with dairy products had both high and low BMFGM titres.

3.0 COMPARISON OF ASSAY METHODS USED AT BATH AND CARMARTHEN

3.1 Comparison of ELISA techniques.

The results discussed in Section 2 show no significant differences between IgG anti-BMFGM antibody levels of control and MI patients (a possible difference may be detected at very low values, but would suggest an increase in titre of control samples, see Sect 2.3, p117). At the time of these studies, Rees and Thomas, in Carmarthen were finding an elevation in antibody levels in MI patients compared with controls (Rees and Thomas, personal communication).

It seemed appropriate, therefore, to compare the assays used at Bath and Carmarthen. The assay used by Carmarthen differed from the Bath assay in two major respects:-

1. The antigen used was a Dried Milk extract [previous work however, had shown titres using this to give an excellent correlation with those obtained using BMFGM as an antigen (Rees,1985)].

2. The conjugate used was commercially produced goat

anti-(human polyvalent immunoglobulin) antibodies which would detect IgG, IgA, and IgM.

35 samples were assayed by using the Bath ELISA system (see Sect 1.1, p101) but varying the antigen and conjugate as follows:

i). No change, i.e. original Bath assay.

ii). Dried milk extract as antigen (see Methods Sect 3.3 (i), p87), in place of BMFGM.

iii). Carmarthen conjugate, in place of the Bath conjugate, at a dilution of 1:1000.

iv). A combination of ii and iii above i.e. dried milk extract as antigen and Carmarthen conjugate. This is essentially the Carmarthen assay.

The mean values of 5 separate experiments were recorded. The results are compared in Fig 36a (i v ii), Fig 36b (i v iii), and Fig 36c (i v iv), p131, and show highly significant correlations in all three cases (Rs = 0.976, 0.942, and 0.936, respectively. Values greater than 0.325, p=0.05, show a significant correlation). The effect of changing the conjugate was greater than that resulting from variation in antigen.

Another cruder way of comparing data was explored. Titres were classified as low, medium and high and the mean was calculated for each:-

High titres are where, x > or =, 70% Std,

Medium titres, 70 > x < 10% Std and

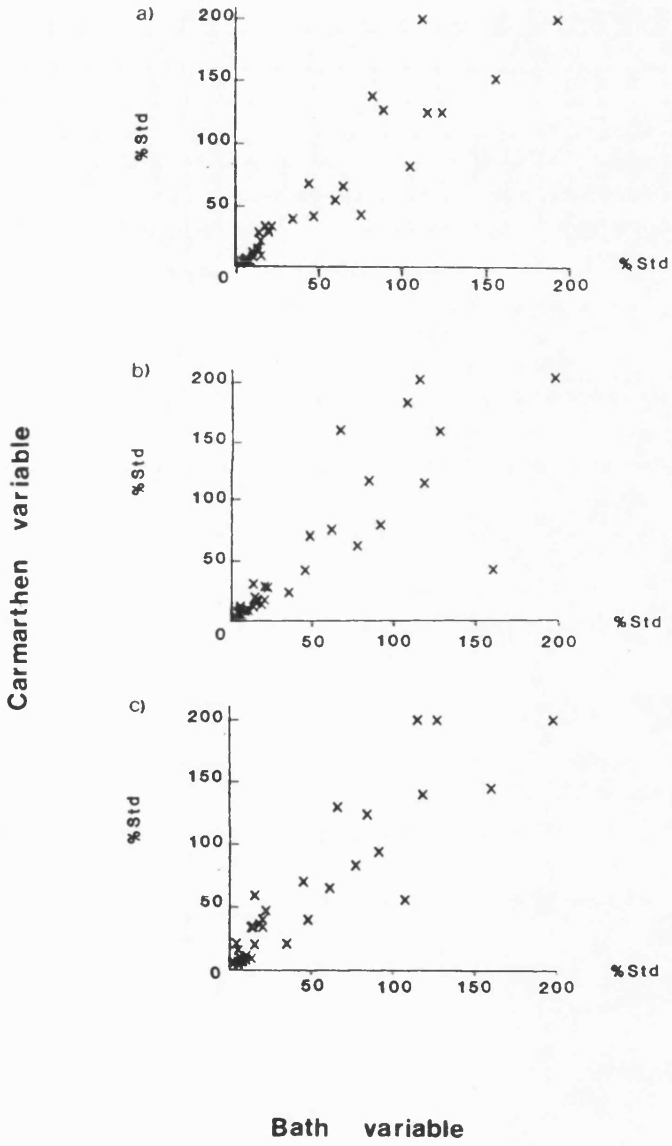
Low titres, where x < or = 10% Std

Fig36 A comparison of:-

a) Bath and Carmarthen antigens, using Bath conjugate.

b) ,, ,, ,, conjugates, using Bath antigen.

c) ,, ,, ,, assays.



Again, changes in conjugate seemed to have more effect than changes in antigen (Table 18, p133).

A series of 5 sera, used by the Carmarthen group as standards in their assay, were provided. They were assayed using BMFGM as antigen, and with both conjugates. The results are shown in Table 19, p133. Again, this shows that the use of different conjugate rather than antigen is causing discrepancies between results obtained at Bath and at Carmarthen.

3.2 Effect, of freeze-thawing of sera, on IgG, IgA, IgM and Polyvalent anti-BMFGM titres.

An alternative explanation for differences between results obtained at Bath and Carmarthen could lie in differences in the number of times that sera had been frozen and thawed. Three different sera were divided into 10 aliquots (0.5 ml). Nine of the aliquots were frozen at -40°C (approx 45 min), the 10^{th} aliquot was reserved for analysis of an unfrozen sample. The frozen aliquots were allowed to thaw at room temperature (approx 15 min),and another aliquot of each serum was retained while the remaining eight aliquots of each serum were re-frozen at -40°C as above. The process was continued until 10 samples (frozen and thawed 0-9 times) were available for each of the three sera. IgG, IgA, IgM, and Polyvalent anti-BMFGM antibodies were detected using commercially produced conjugates, specific for each antibody type. Fig 37, p134, shows that freezing and thawing appears not to affect the titre.

Table 18 Average values for high, medium, and low titre samples in the BATH ELISA compared with the average values of their corresponding titres when varying antigen and conjugate

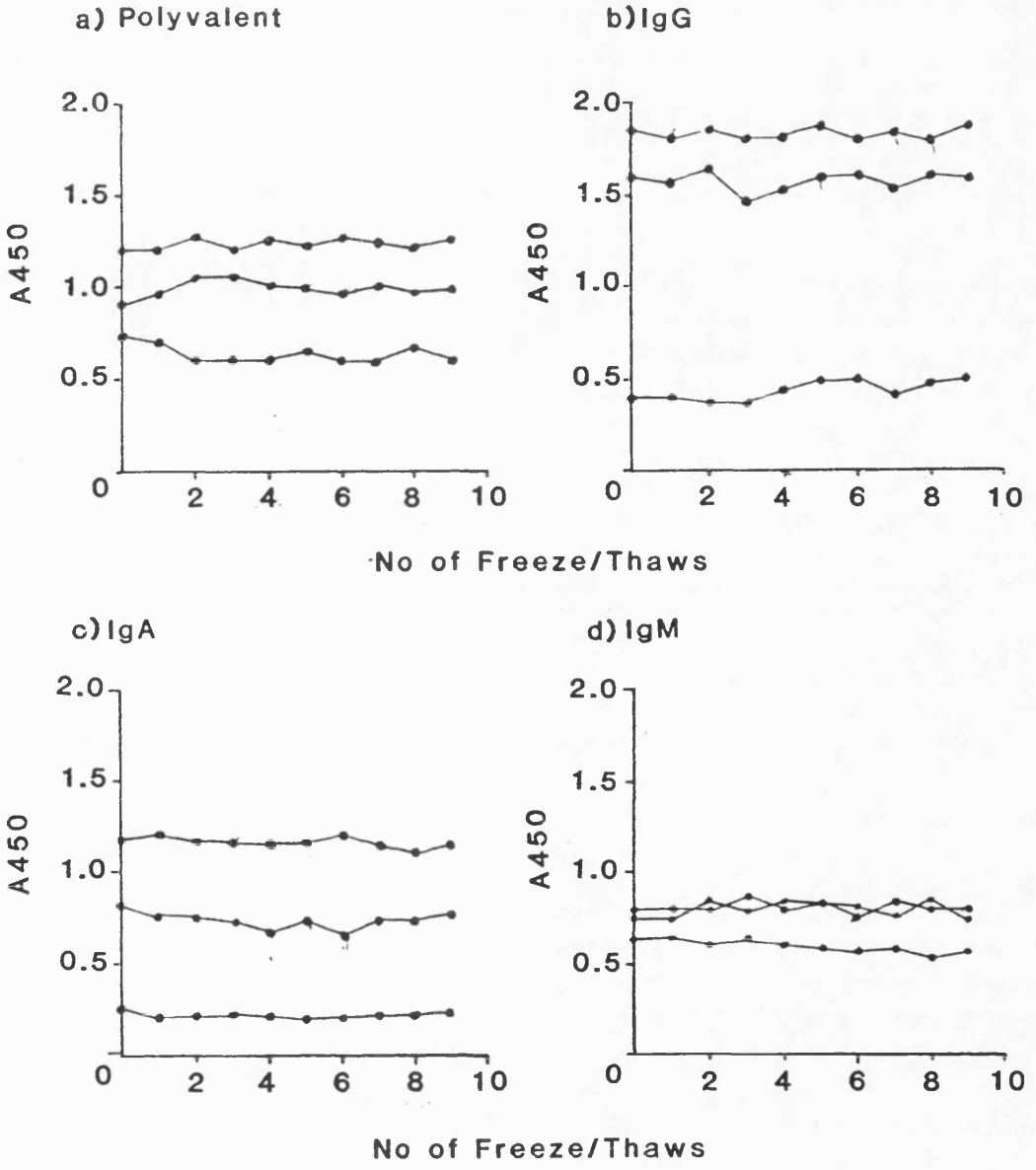
Antigen	BMFGM		DRIED MILK	
Conjugate	BATH	CARM.	BATH	CARM.
Titre				
HIGH	120.0	127.3	121.3	127.0
MEDIUM	28.9	39.6	32.8	45.6
LOW	5.7	8.1	5.8	8.8

Table 19 Effect of conjugate on titre of Carmarthen standards, using BMFGM as antigen.

Conjugate Source		
Carmarthen	Bath	Carmarthen
Titre, % Carm. Std quoted by Carm.	Titre, % Carm. Std, calculated at Bath.	
84	93.3 ± 5.6	77.6 ± 9.4
43	20.9 ± 2.3	46.7 ± 3.8
21	8.3 ± 1.4	19.0 ± 2.1
20	15.0 ± 3.4	21.0 ± 3.5
14	6.8 ± 2.2	16.0 ± 1.8

(n = 5)

Fig 37 The effects of freeze thawing on anti-BMFGM antibody levels with respect to; a) polyvalent, b) IgG, c) IgA, and d) IgM antibodies.



Standard Errors < 5% Absorbance value (n = 3).

3.3 Analysis of the laboratory prepared goat anti-human IgG conjugate.

The conjugate prepared in the laboratory was compared to a commercially available conjugate specifically raised to IgG (i.e. directed against the Fc portion of IgG). Fig 38, p136, shows that while both conjugates detect IgG molecules, the Bath conjugate detects an F ab' fragment whereas that of the commercial conjugate does not i.e. Bath conjugate is possibly detecting light chain as well as heavy chain fragments.

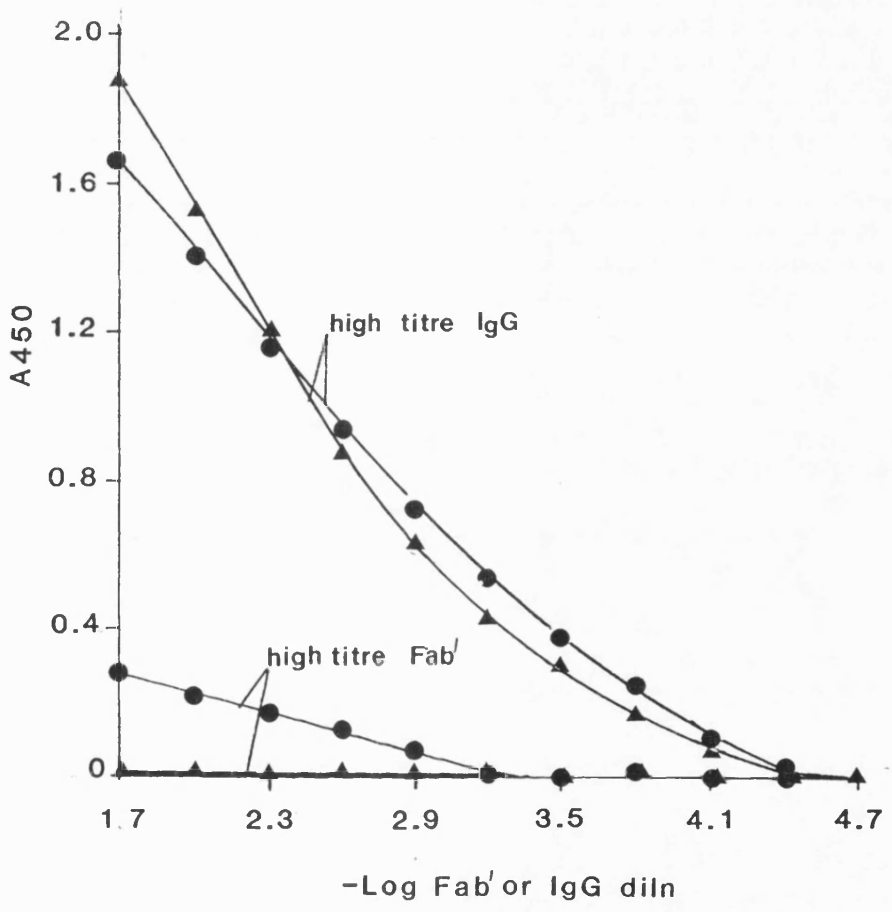
4.0 Analysis of IgG, IgA, IgM, and Polyvalent anti-BMFGM antibodies in MI patients and controls.

4.1 Preliminary analysis

Fig 39, p137, shows the results of titration of the standard sera, using the BMFGM ELISA, with different conjugates (goat anti-human IgG, IgA, IgM and Polyvalent conjugated to HRP (1:1000 in PBS/Tween 0.05%). It can be seen that slightly lower dilutions of sera are necessary to detect IgA, and IgM, compared with IgG. Nevertheless, it is clear that IgG, IgA, and IgM molecules are being measured in the serum. In subsequent assays, IgG and Polyvalent antibodies were measured at 1:200 dilution of serum, IgM, and IgA were measured at 1:50 dilution of serum.

Two sets of 65 control and 65 "New" MI patients were chosen randomly and assayed for the above antibodies, see Figs 40 (a-d), p139-143. A K-S analysis was

Fig 38 Analysis of the laboratory prepared conjugate.



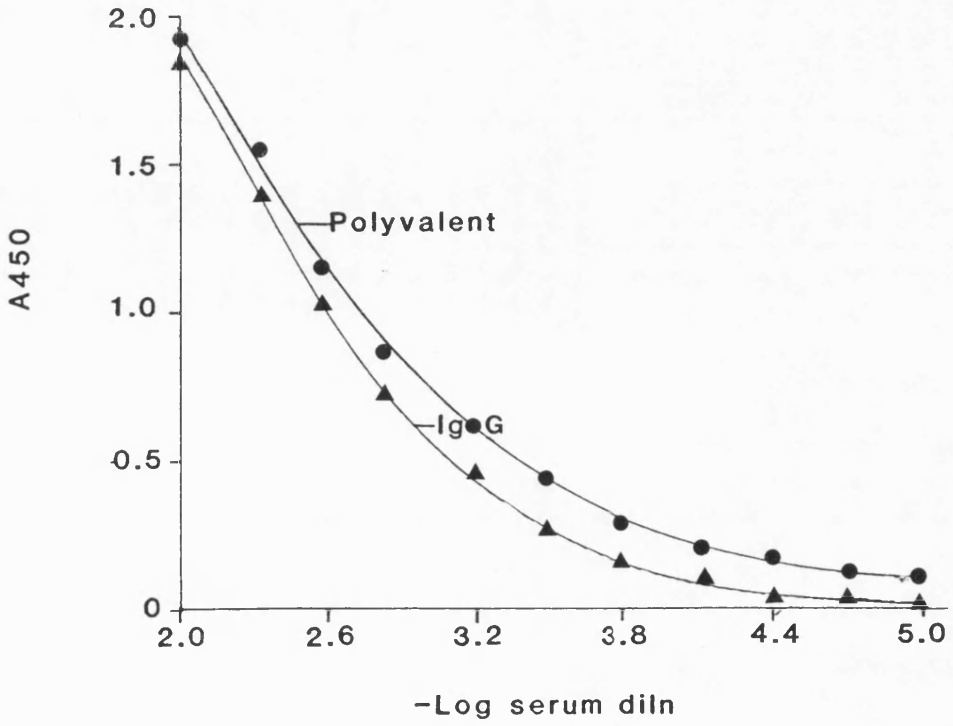
▲ = Commercially available anti-human IgG

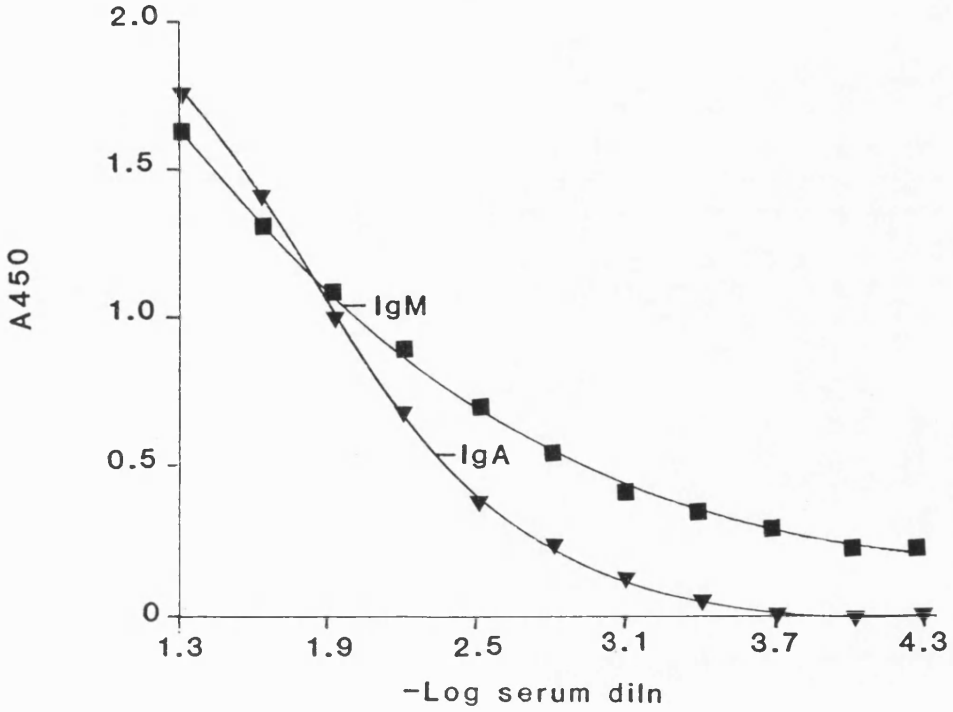
● = Anti-human IgG prepared in the laboratory

Fig 39 Standard curves produced from the standard sera already in use (BMFGM ELISA Results Sect 1.1) but using:-

a) anti-polyvalent, anti-IgG, and

b) anti-IgA, anti-IgM, commercially available conjugates.





performed on each set of results. There was no significant difference between MI and control titres of Polyvalent, IgG or IgA anti-BMFGM antibodies, but IgM antibodies were significantly raised in MI patients compared to controls.

[For polyvalent anti-BMFGM antibody detection the greatest difference between MI and control samples occurred above 5-10% of the Std (65.1% of MI and 55.5% of control had values greater than 9.9% of the Std in set 1, and 65.1% and 48.5% respectively in set 2). K-S values of 0.136 and 0.167 were calculated for each set respectively, 0.237 is the minimum value necessary to show a significant difference at $p=0.05$.

For IgG comparisons, the greatest difference between the two populations occurred at 5-10% of Std (53% of MI, and 59% of controls had values greater than 9.9% of Std in set 1, and 53% and 58% respectively in set 2). K-S values of 0.061, and 0.045 were calculated for set 1 and set 2, values above 0.237 being necessary for a significant difference.

For IgA, the greatest difference occurred above 30-40% of the Std (21.2%of MI and 7.6% of control in set 1 and 22.7% and 9% in set 2). K-S values for set 1 and set 2 were both 0.136; 0.237 or above is necessary to show a significant difference.

For IgM, the greatest difference between the two populations occurred above 10-20% of the Std (82% of MI and 44% of control,in set 1 and 85% and 60% respectively in set 2), giving K-S values of 0.379 and 0.257 for sets

Fig 40 Distribution of anti-BMFGM antibodies in two sets of 65 "New" MI patients and 65 controls, measuring:-

a) Polyvalent

b) IgG

c) IgA

d) IgM antibodies

I - Set 1 samples

II - Set 2 samples

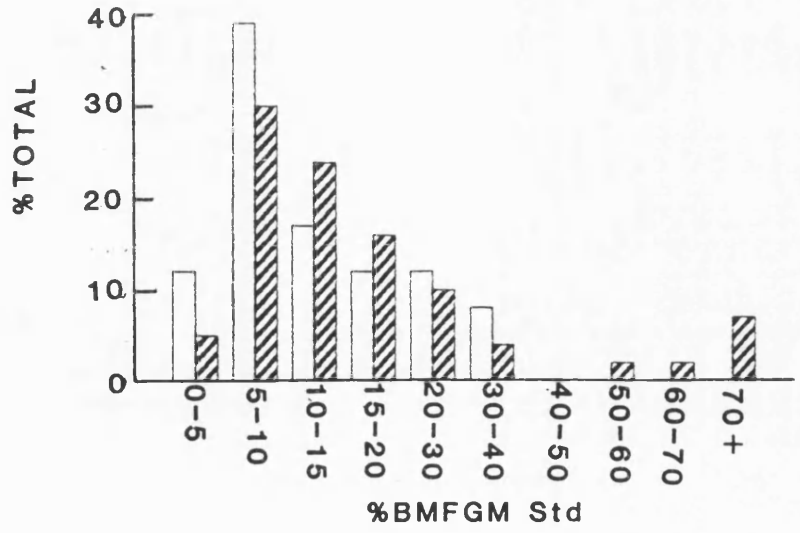
Key:-

 = Control

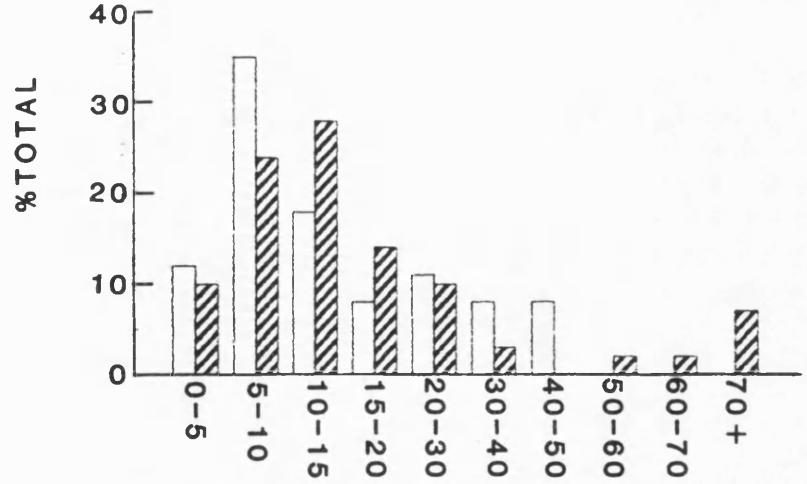
 = MI

a) Polyvalent

I

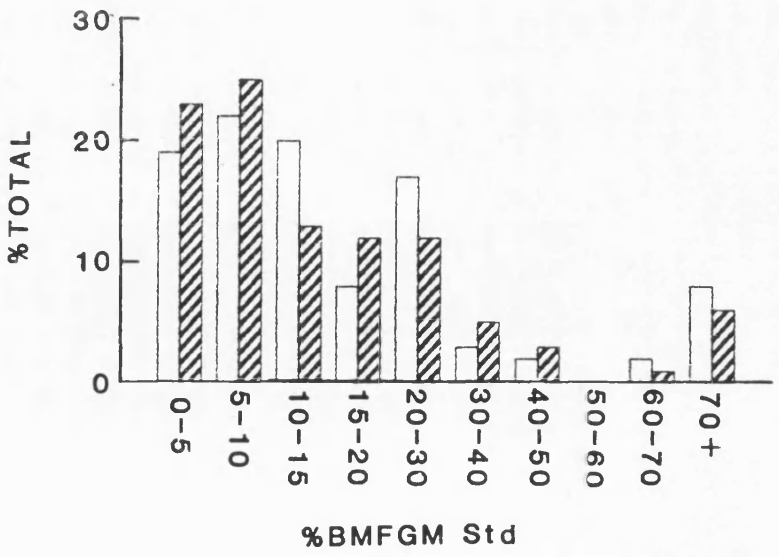


II

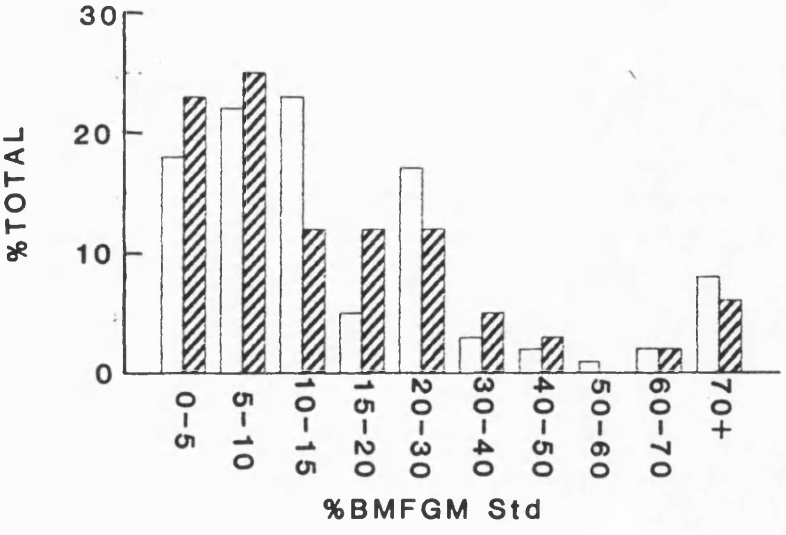


b) IgG

I


30
20
10
0
%TOTAL
0-5
5-10
10-15
15-20
20-30
30-40
40-50
50-60
60-70
70+
%BMFGM Std

II


30
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10
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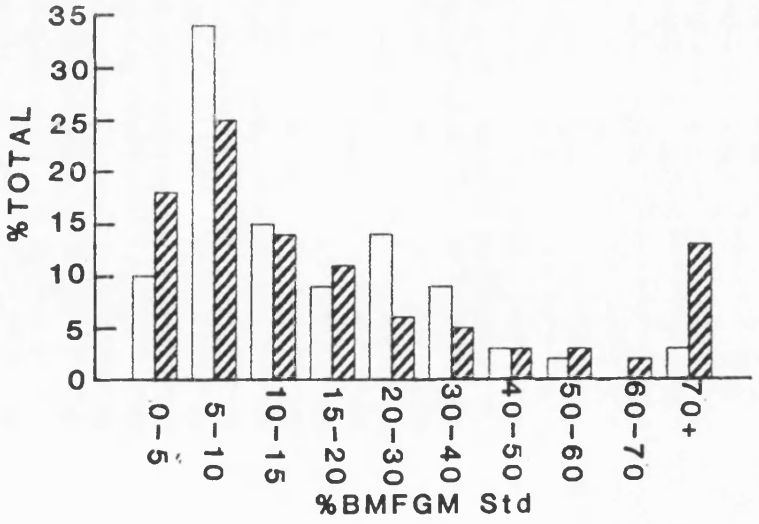
%TOTAL
0-5
5-10
10-15
15-20
20-30
30-40
40-50
50-60
60-70
70+
%BMFGM Std

I

c) IgA


35
30
25
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15
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5
0
%TOTAL
0-5
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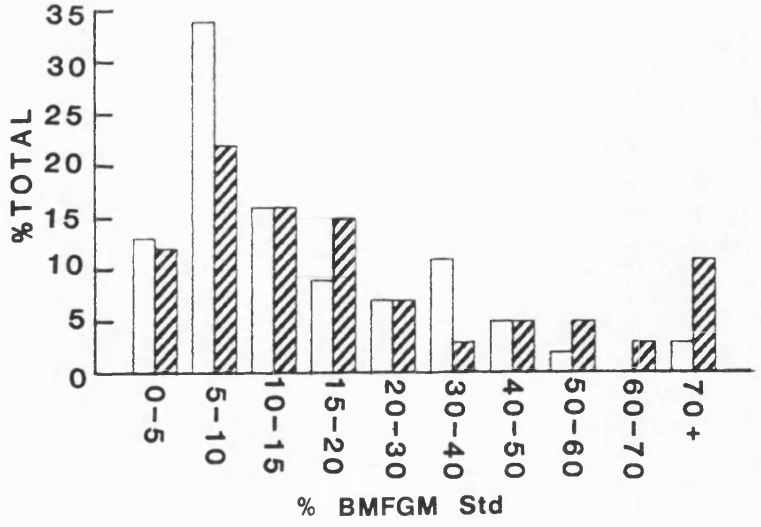
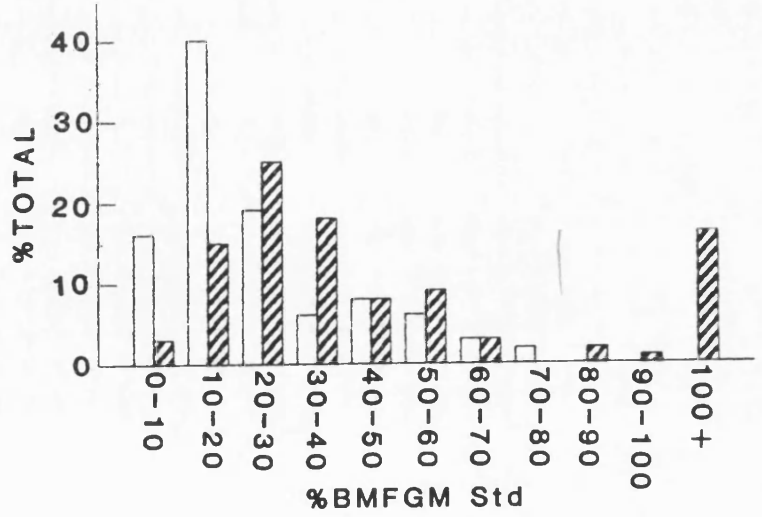
10-15
15-20
20-30
30-40
40-50
50-60
60-70
70+
%BMFGM Std

II

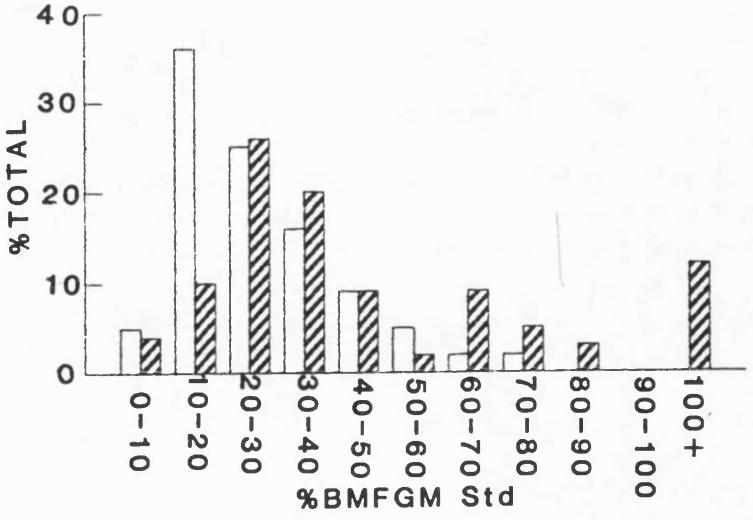

35
30
25
20
15
10
5
0
%TOTAL
0-5
5-10
10-15
15-20
20-30
30-40
40-50
50-60
60-70
70+
% BMFGM Std

I

d) IgM


40
30
20
10
0
%TOTAL
0-10
10-20
20-30
30-40
40-50
50-60
60-70
70-80
80-90
90-100
100+
%BMFGM Std

II


40
30
20
10
0
%TOTAL
0-10
10-20
20-30
30-40
40-50
50-60
60-70
70-80
80-90
90-100
100+
%BMFGM Std

1 and 2, both values of which are above the value of 0.237 necessary to show a significant difference between MI and control samples.]

4.2 Assay of 100 v 100 MI sample with controls

The next investigation was to compare a larger set of MI patients and controls. This time however, the MI patients were selected from a specific area, that of the South Wales Valleys, which was most compatible with the area of collection of the controls (Caerphilly and Speedwell). As all control samples were from men of < 65 years of age, all MI samples > 64 years of age were excluded.

The results of measuring Polyvalent, IgG, IgA, and IgM anti-BMFGM antibody levels are shown in Figs 41 (a-d), p145-147. As in the previous section (4.1, p135), no significant differences were observed when considering polyvalent, IgG or IgA antibodies. Differences were however, significant with IgM.

[For polyvalent antibody measurement, the greatest difference between MI and control samples occurred above 5-10% of the Std (70% of MI and 56% of controls).A K-S value of 0.14 calculated was below the value of 0.192 necessary to show a significant difference at p=0.05.

For IgG, the greatest difference occurred above 5-10% of the Std (52% of MI and 63% of control samples were above this). A K-S value of 0.11 was below the value of 0.192 necassary for significance at p=0.05.

For IgA, the greatest difference occurred above 0-5.0%

Fig 41 A comparison of anti-BMFGM antibodies in 100 MI samples sets of 100 "New" MI samples (region and age matched), with and 100 controls, measuring:-

a) Polyvalent

b) IgG

c) IgA

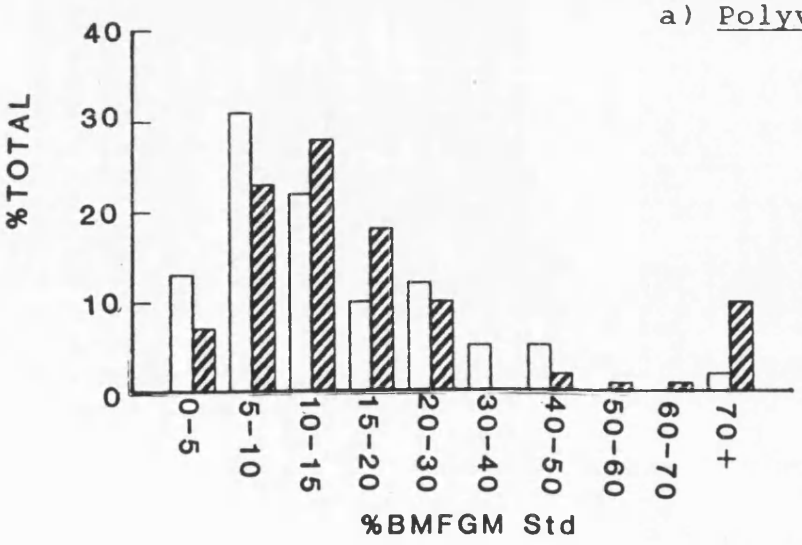
d) IgM antibodies

Key:-

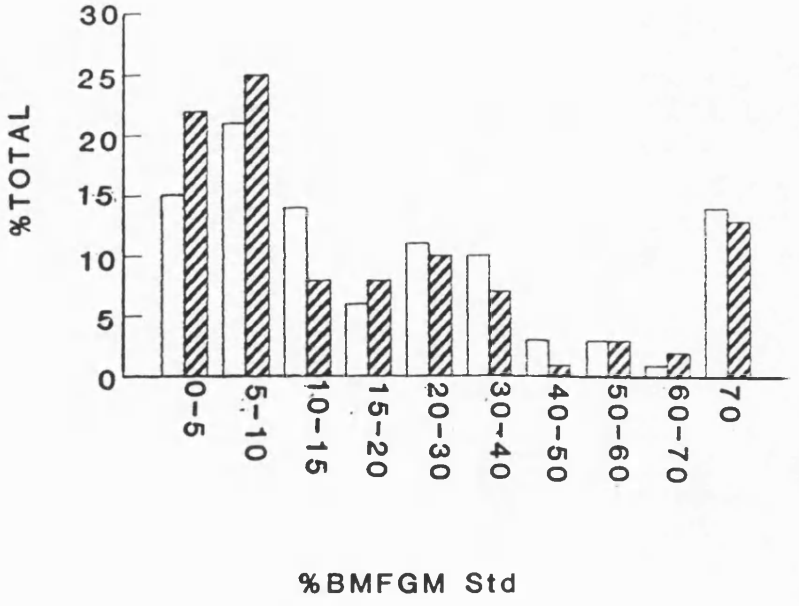
 = Control

 = MI

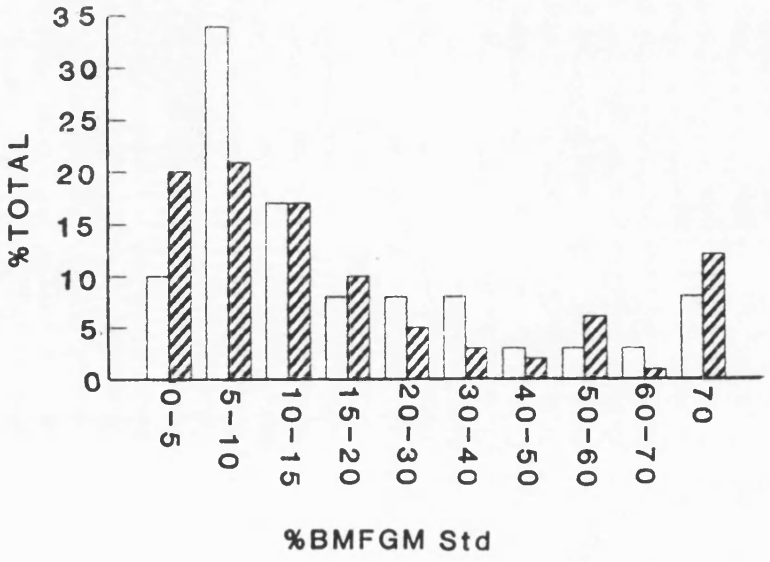
a) Polyvalent



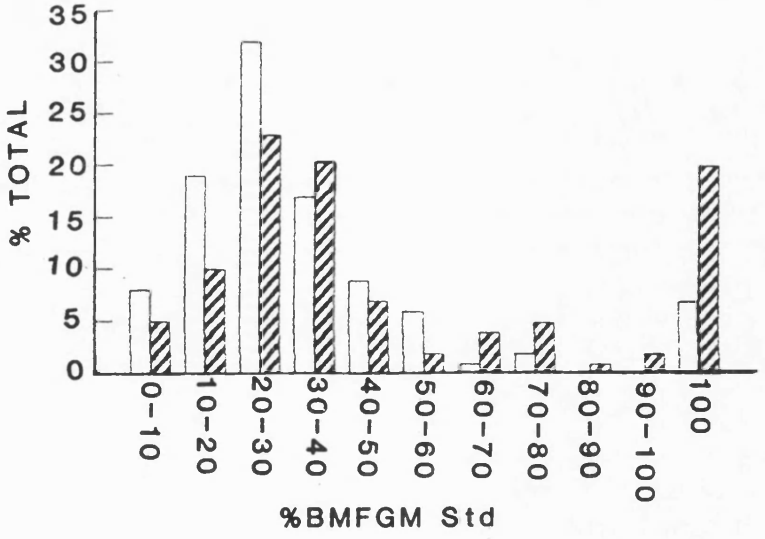
b) IgG



c) IgA


35
30
25
20
15
10
5
0
%TOTAL
0-5
5-10
10-15
15-20
20-30
30-40
40-50
50-60
60-70
70
%BMFGM Std

d) IgM


35
30
25
20
15
10
5
0
% TOTAL
0-10
10-20
20-30
30-40
40-50
50-60
60-70
70-80
80-90
90-100
100
%BMFGM Std

of the Std (79% of MI and 91% of control samples were above this). A K-S value of 0.12 was again below the necessary value of 0.192 to show significance.

For IgM, the greatest difference occurred above 50-60% of the Std, although the difference above 20-30% of the Std was only slightly lower, (32% of MI and 10% of controls had values of greater than 59% of the Std). A K-S value of 0.220, greater than that necessary to show significance, 0.192 at P=0.05, indicated a significant elevation of IgM anti-BMFGM antibodies in MI patients compared to controls.

5.0 ANTIGENIC DETERMINANTS OF THE BMFGM

5.1 Affinity purification of anti-BMFGM antibodies

To facilitate characterisation of antigenic determinants of the BMFGM, serum anti-BMFGM antibodies were affinity purified. These were then used as tools in further immunochemical analyses (see Results Sect 5.2, p160, 6.2.1, p197 and 6.4, p210).

a) Batch adsorption method

This method had been described by Freed (personal communication). It entailed mixing BMFGM with serum, centrifuging to remove BMFGM, adding 0.05M Glycine/HCl buffer pH 2.8 to the BMFGM, which was removed leaving the affinity-purified antibodies in Glycine/HCl buffer. Experimental details were sparse, so the following protocol, based on the above, was used initially.

A suspension of BMFGM in pooled human serum, was incubated at 37°C and centrifuged (100,000 g, 1h). To avoid any non-specific binding of antibodies or other proteins to BMFGM, a wash step was included at this point. BMFGM was resuspended in PBS/0.75M NaCl and centrifuged as before. The BMFGM pellet was then resuspended in 0.05M Gly/HCL, pH 2.8 for 1h at 37°C, and centrifuged as above. The supernatant was decanted, neutralised, by the addition of 4M NaOH and dialysed against a large volume of ammonium acetate (0.1%) at 4°C overnight. The dialysed solution was then lyophilised, and redissolved in PBS.

To maximise anti-BMFGM binding to the BMFGM, the following conditions were investigated:-

1. The amount of BMFGM required to absorb out total anti-BMFGM activity was investigated. Varying amounts of membrane were incubated for 1h at 37°C with three different sera, of varying titre, to discover how much BMFGM was required to completely absorb out anti-BMFGM antibodies

Fig 42, p150, shows that for a very high titre serum 25mg / ml of sera will absorb out all anti BMFGM antibodies.For a mid-titre and low titre serum the amount of BMFGM required to produce the same effect was less. For subsequent experiments 25mg of BMFGM/ ml of sera was used.

2. The time for maximal binding of antibody to BMFGM was investigated. BMFGM was incubated with serum (25mg/ml),

Fig 42 Absorption of anti-BMFGM activity in 3 different sera by the addition of varying amounts of BMFGM.

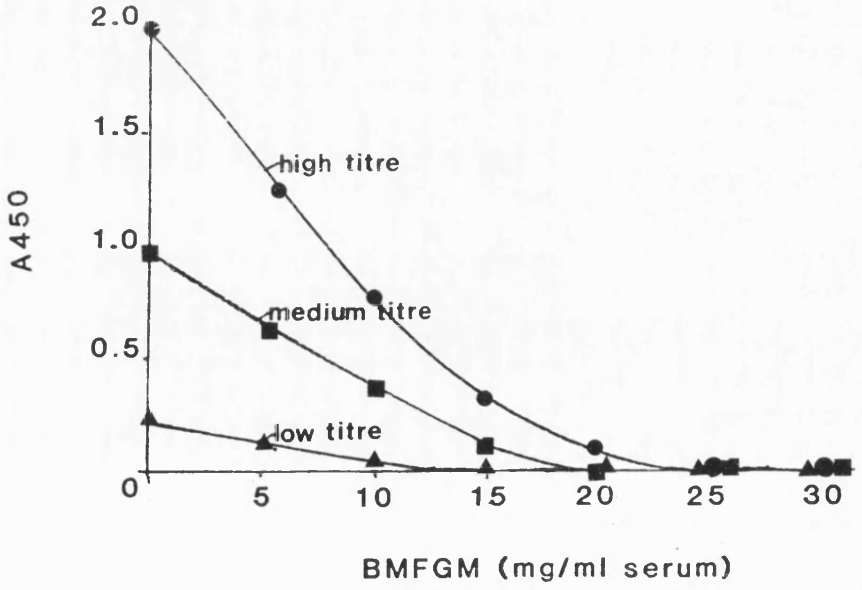
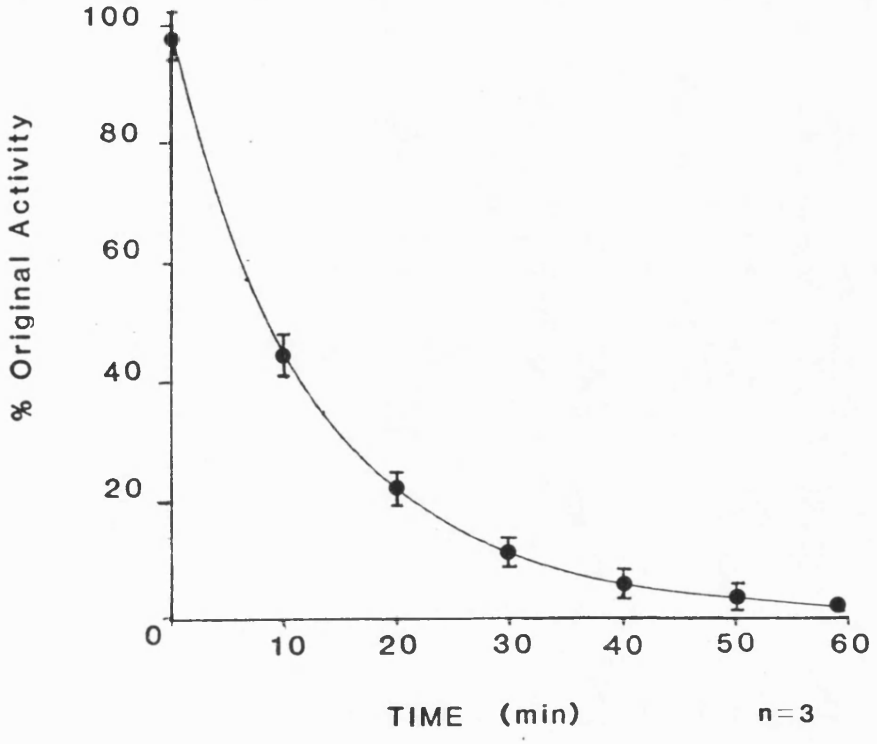


Fig 43 Optimisation of time required for maximal binding of anti-BMFGM antibody to BMFGM.



by stirring at 37°C. At various time intervals (0-60min), an aliquot (5ml) of suspension was taken, centrifuged and treated as for the method outlined above.

Fig 43, p150, shows that almost all (95%) antibody has bound to the BMFGM after 50 min, under the conditions used for incubation. For further experiments an incubation time, for BMFGM with sera, of 60 min, was allowed.

The activity recovered by the above process was poor (0.1 ± 0.1% of original activity, n=3).

The following experiments were designed to try to optimise recovery of anti-BMFGM antibodies from a pooled, high titre serum.

1. The volume of Gly/HCl used in incubation of antibody bound BMFGM was varied 100-fold (1-100ml). This had no significant effect on activity recovered (0.1 ± 0.1% orig. serum., n=3).
2. The time of incubation of Gly/HCl with BMFGM was varied between 1h and 24h. This also had no effect on recovery of activity (0.1 ± 0.1% orig.serum., n=3).
3. It was considered possible that the denaturing agent Gly/HCL may have been interfering with either the activity of the antibody itself, or the antigenic structure of the BMFGM. Incubation of serum with Gly/HCl (37°C, 1h), had the effect of reducing the activity of the sera to 85.3 ± 7.2% of the original, n=3. The activity of the sera was, in fact, reduced by

incubation of the sera at 37°C, 1h (to 95.4 ± 5.5% of original activity). A similar experiment was performed, treating BMFGM with Gly/HCl. BMFGM was used to coat ELISA plates (as in Results Sect 1, p101), after incubation with and without Gly/HCl (37°C, 1h). Treatment of BMFGM at 37°C alone had no effect on the antigenicity of the membrane (100 ± 6.1% original),and treatment with Gly/HCl reduced the activity of the membrane to 91.6 ± 4.3% of original activity, (n=3). The effect of Gly/HCl on both BMFGM and sera did not reduce the activity of either sufficiently to account for the poor recoveries of anti-BMFGM antibody.

4.The eluting agent was varied. In place of Gly/HCl, 3M-potassium thiocyanate, 0.1M-sodium borate, pH10, and 0.05M-ammonium hydroxide, pH11 were investigated. Some improvement was noted using ammonium hydroxide (0.3 ± 0.1% original, n=3) therefore this was the choice eluting agent in all subsequent experiments. Potassium thiocyanate, and sodium borate showed no improvement over Gly/HCl (0.1 ± 0.1% original activity, n=3).

The final experimental protocol for affinity purification of anti-BMFGM antibodies by a batch absorption method is described in Materials and Methods Sect 2.9, p81.

b) Design of an affinity column

An affinity column was prepared by a combination of the methods of Sela and Edelman, 1977; and Steiner 1985, as

follows.

The column matrix, Sephadex G-50 (50ml) was stirred at room temperature with Concanavalin A (Con A) (50mg in PBS). Excess Con A was removed by decantation and the ConA- Sephadex was stirred with a BMFGM suspension (5ml, 0.1mg/ml) overnight at 4°C. This mixture was degassed and poured into a column (2.5 x 25cm). The column was washed for 30min with glutaraldehyde (30% in PBS, at 30ml/h for 2h) and then equilibrated with PBS. Before passing serum through the column (10ml,30ml/h), 1% BSA in PBS was passed through, (1h, 30ml/h). This was followed by exhaustive washing with PBS until the optical absorbance of the column effluent, measured at 280nm, was < 0.005. Material bound to the column was then eluted in 0.5M ammonium hydroxide pH11, at 40ml/h. The eluate was immediately adjusted to pH 7 with 2M-acetic acid, and lyophilised, giving anti-BMFGM antibodies representing $5.2 \pm 3.4\%$, n=3 of those loaded onto the column.

A second commercially-available matrix, Sepharose-4B, to which Con A was already bound, was used in place of Sephadex G-50 (5ml instead of 50ml). Preparation of the affinity column and purification then proceeded in the same way, using the same quantities of serum. Using this matrix, activity recovered was $12.2 \pm 4.4\%$ of the original activity n=3.

The above experiment was repeated except that a wash step was included to remove non-specifically bound material from the column. This buffer contained PBS,

0.75M NaCl, 0.2M α-methylmannoside and 0.2M α-methyl glucoside. Table 20, p155 shows a purification profile of anti-BMFGM antibodies from serum.

The wash step acts to remove some BMFGM activity, 5.3 ± 2.6% of original activity, but this does not detract from the activity recovered in the final affinity purification stage (14.2 ± 3.6 %, n=3). Table 20, also shows the control experiment of passing PBS through the column in place of serum. This shows that no anti-BMFGM activity is recovered at any of the stages. Some protein recovered after the wash stage may be due to the BSA bound to the column.

The following experiments were designed to optimise recovery of anti-BMFGM antibody from the affinity column using a batch of pooled human sera.

1. Serum was passed through the affinity column, once, twice, and recirculated through the column overnight. Repeated passage through the column resulted in an increase of recovered activity from 10.2 ± 4.3 % to 15.8 ± 2.7 % of the original activity.

2. A second cross-linking agent, dimethylsuberimidate, used to covalently attach BMFGM to the column, was investigated. The method of preparation was according to Lang et al (1982). Dimethylsuberimidate (0.17-0.2 mg/ml) in 0.2M triethanolamine, pH 8.4 was passed through the BMFGM – ConA Sepharose column, in place of glutar-aldehyde. After 3h, the reaction was terminated by recirculation through the column overnight of 0.5M

Table 20 Affinity purification of anti-BMFGM antibodies from serum using an affinity column (see text), compared with PBS as a control.

Column Eluate	Source for purification			
	SERUM		PBS	
	Activity (%)	Protein (mg)	Activity (%)	Protein (mg)
Orig.	100	82.2 ± 5.6	0	0
N B	28.2 ± 6.6	78.4 ± 8.2	0	0
N S B	5.3 ± 2.6	8.1 ± 4.2	0	4.3 ± 1.8
S B	14.2 ± 3.6	0.02 ± 1.0	0	0

n = 3

Key to above table:-

Orig. - Original serum applied to column

N B - Material not binding to column

N S B - Non-specifically binding material eluted after washing with salt

S B - Specifically binding material eluted after Ammonium Hydroxide treatment

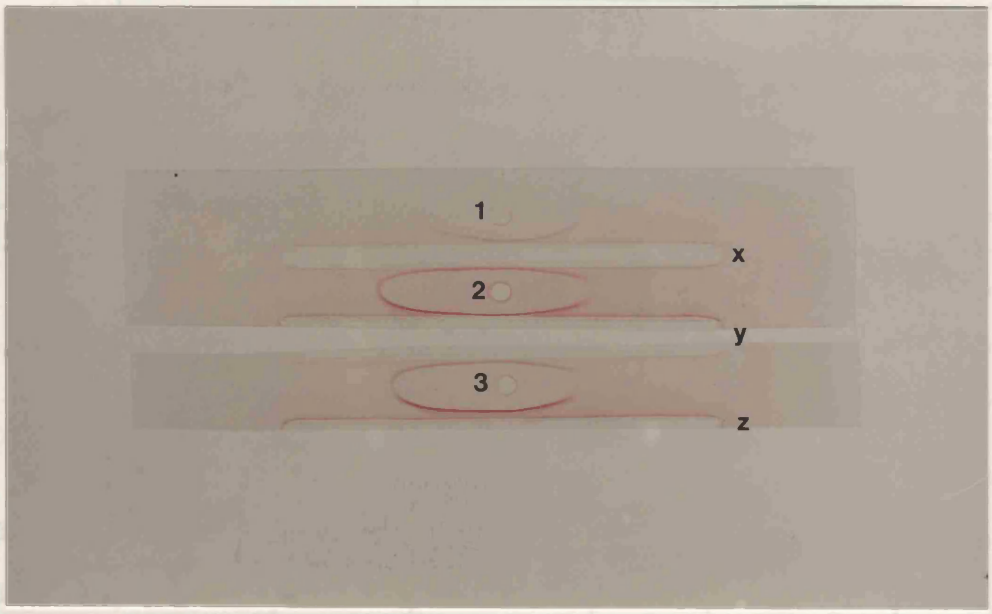
lysine, in the same buffer. Activity recovered was increased from 14.7 ± 3.3 % (using glutaraldehyde as a cross-linking agent) to 20.4 ± 3.6 % of the original antibody activity (n=3), when dimethylsuberimidate was used.

3. Immunoelectrophoresis of the affinity purified anti-BMFGM antibodies had shown that they were largely IgG. Anti-(human polyvalent) antibody gave a very similar result compared to anti-(human IgG) when used to detect affinity purified anti-BMFGM antibodies), see Fig 44, p 157. When serum (1ml) or the equivalent amount of IgG (10mg,approx) was applied to the column, the final recovery of activity was similar (13.9 ± 2.6% for serum, and 14.5 ± 2.0% for IgG, n=3).

In all further experiments, IgG was used as the source of antibodies for purification.

In all of the experiments outlined, antibody activity of 25-35% was lost in the flow-through serum. Attempts were made to optimise the amount of IgG applied. The results (Table 21, p158) show that the anti-BMFGM activity present in the flow through does not result from an excess of anti-BMFGM antibody. There appears to be an anti-BMFGM antibody population which does not bind to the column. This is indicated by the similar amounts of activity in the flow through from the application of 2-16 mg IgG/ml of PBS. However, on analysis of the affinity purified fraction,the optimum loading of IgG appears to be 10mg/ml of PBS.

Fig 44 Immunoelectrophoresis of two preparations of affinity purified anti-BMFGM antibody.



Well No

1. Human IgG
2. Affinity anti-BMFGM I
3. Affinity anti-BMFGM II

Trough

X. Goat anti-(human IgG)

Y. Goat anti-(human polvalent antibodies)

Z. Goat anti-(human IgG)

Table 21 Optimisation of antibody purification from varying amounts of IgG, prepared from a pooled serum, (per 5 ml affinity column).

IgG (mg/ml)	N B		N S B		S B	
	Act. (% Orig)	Prot. (mg)	Act. (% Orig.)	Prot. (mg)	Act. (% Orig)	Prot. (mg)
2	23.2	1.2	2.1	3.1	8.5	0.05
4	28.5	3.8	5.2	3.2	10.6	0.09
6	26.1	5.5	4.8	4.4	15.4	0.17
8	25.9	7.6	3.7	6.5	20.1	0.20
10	32.9	9.6	6.8	6.2	19.7	0.24
16	33.1	15.5	9.4	4.2	22.9	0.25

(NB, NSB and SB, as for Table 20 ,p 155)

Table 22 Results of affinity purification of 10 different human sera, and an immune sera raised in rabbit to BMFGM (RABMFGM).

SERUM No	BMFGM titre (% Std)	Recovery (% Orig)	Prot. (mg)	BMFGM Ab level (mg/ml serum)
1	100 (Pooled)	20.6	0.24	1.17
2	123	21.4	0.19	0.89
3	89	19.6	0.20	1.02
4	82	18.3	0.16	0.87
5	46	15.6	0.14	0.90
6	32	16.1	0.10	0.60
7	20	14.3	0.06	0.40
8	10	9.9	0.04	0.40
9	5	12.3	0.01	0.08
10	2	13.2	0.01	0.07
RABMFGM		31.6	0.69	2.18

The final protocol for preparing a column and purifying anti-BMFGM antibodies is described in Materials and Methods Sect 2.9, p8.2.

Table 22, p159 shows the results of affinity purifications from 10 different human sera, of varying titres, and cf anti-serum to BMFGM raised in rabbit. It can be seen that the % recoveries of the affinity fraction are similar in all, apart from the rabbit anti-BMFGM sera which shows a recovery of 31.6% . The difference between titres is represented by the protein recovered in each i.e. high titre sera have a higher recovery of protein associated with the affinity fraction. Assuming 100% activity in the original serum it is possible to estimate the total amount of anti-BMFGM specific IgG present per ml of each individual serum, the results of which are also shown in Table 22.

5.2 Western blotting of SDS-PAGE patterns of BMFGM, followed by immunoblotting.

BMFGM, prepared as described in Materials and Methods (Sect 2.1, p70), was subjected to SDS-PAGE and then polypeptides were transferred to nitrocellulose sheets ,Materials and Methods (Sect 4.1 and 4.2, p89, 93). The transferred bands were incubated with either serum (1:50), or IgG (1:200), prepared from it, followed by an appropriate conjugate (1:1000), Materials and Methods (Sect 4.2, p94).

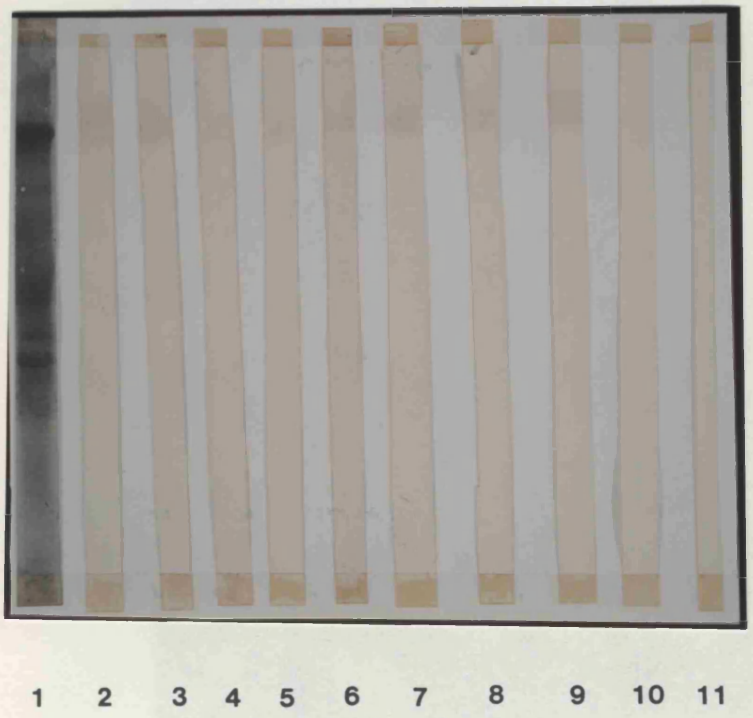
Figure 45, p162, shows the results of immunoblotting of 9 different human sera. One band at approximately

150 kD appears to be consistently present. Fig 47, p163, shows an immunoblot of human sera on the BMFGM in more detail. Although the 150 kD is present in all sera, other bands, at 28, 36, 45, 59, 66, and 170kD occur in some gels. All of the bands are noticeably more intense in the high titre sera. Fig 47, p164, shows the results of immunoblotting of affinity purified IgG anti-BMFGM antibodies from different human sera. Again the band at 150 kD is detectable in all preparations. Bands of MWt 28, and 94 kD are detectable in the pooled sera and a band of 45 kD is seen in the high titre preparations. In the two high titre sera (lanes 1, and 4), the bands of 150 kD are enhanced.

Fig 48, p165, shows the results of immunoblotting of affinity purified rabbit anti-BMFGM antibodies together with the unpurified rabbit IgG and flow-through fractions (containing 25% of the original activity of the IgG). All gels show a band at 150kD. It is noteworthy that most transferred protein bands are also stained, in contrast to results with human sera, indicating that human anti-BMFGM antibodies may be more specific in their binding.

Fig 49, p166, shows an SDS-PAGE pattern of dried whole milk (as used in the Carmarthen assay). Two regions of low MWt proteins are shown , both of which also stain when immunoblotted with sera.

Fig 45 An immunoblot profile of the BMFGM treated with 9 different human sera.



Lane 1 - BMFGM stained for protein

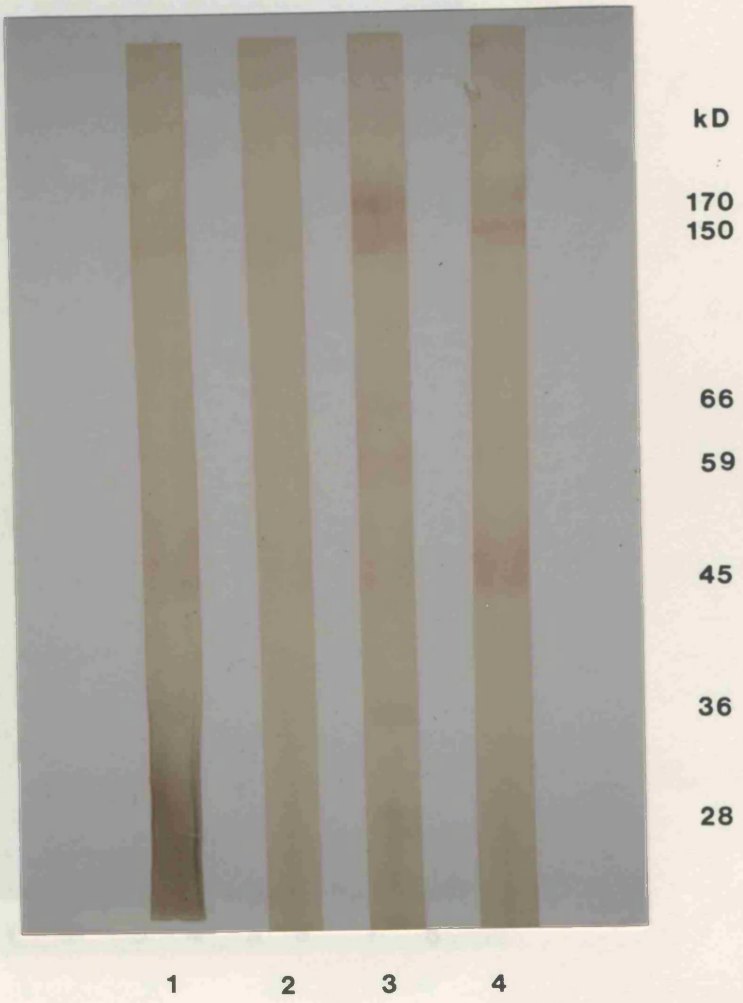
2,3,4,7 and 9 - High titre anti-BMFGM sera

5,6 and 8 - Medium titre sera

10 - Low titre sera

11 - No sera

Fig 46 Immunoblotting profile of human sera against BMFGM.



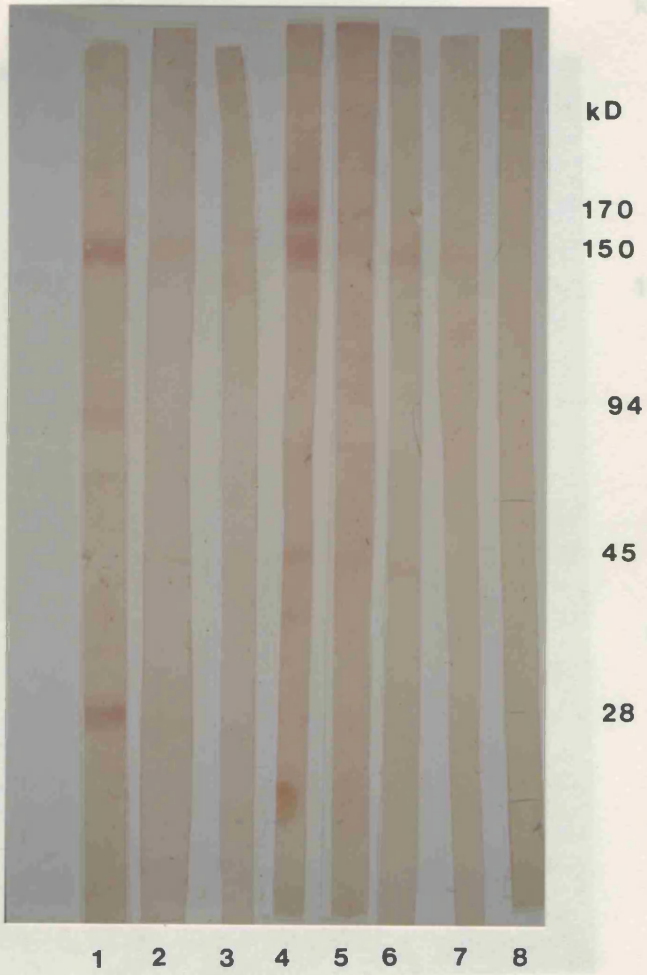
1. Low titre sera

2. Medium titre sera

3,4. High titre sera

(Low, medium, and high, as defined on p130)

Fig 47 Immunoblotting profile of affinity purified anti-BMFGM antibodies against BMFGM.



1. Pooled sera

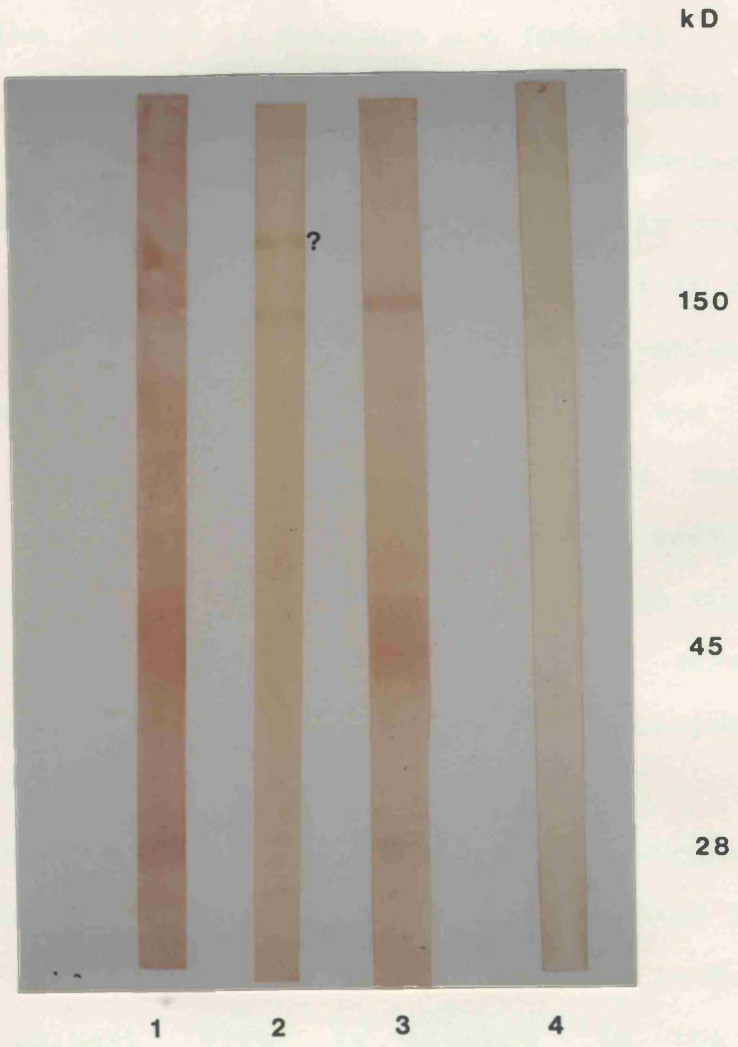
2,3. Low titre sera

4,5. High titre sera

6,7. Medium titre sera

8. No sera

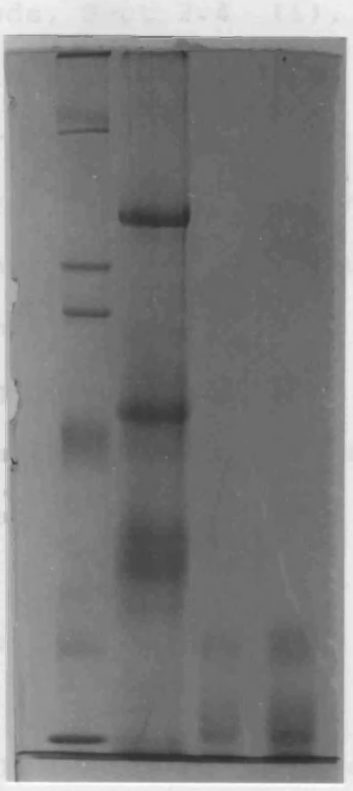
Fig 48 Immunoblotting profile of affinity purified RABMFGM against BMFGM



1. RABMFGM IgG
2. Flow through fraction from affinity column.
3. Affinity purified RABMFGM IgG
4. Blank

? Artefact

Fig49 SDS-PAGE of BMFGM and Dried Milk



L-R

1. SDS MWt markers
2. BMFGM (75µg)
3. Dried Milk (25µg)
4. ,, ,, (50µg)

5.3 Preparation of Xanthine Oxidase for immunochemical analysis of anti-XO antibodies.

XO was initially purified from bovine milk according to the method of Nakamura and Yamazaki, 1982 (Materials and Methods, Sect 2.4 (i), p74). However, of the five preparations made (45.6 ± 10.2 mg protein, 5.29 ± 0.95 IU/mg) from 9L fresh Bovine Milk), only one was suitable for assaying IgG, IgA, and IgM anti-XO antibodies, in an ELISA. The other four preparations caused a background to anti-XO IgM detection see Fig 50, p168. The background was present when sera was assayed, but not in the control with no sera. This background was not eliminated by use of enzyme prepared in the presence of protease inhibitors and was not affected by the proportion of oxidase form of the enzyme (results not shown).

SDS-PAGE profiles of two different preparations of the enzyme, one of which did and one of which did not show a background in IgM ELISA, are shown in Fig 51, p169. A contaminant (83kD) is present in the preparations creating the background to IgM. This was found to be always the case.

Attempts to separate the preparation containing the 150kD and 83kD polypeptides, by gel-filtration on G-200 Sepharose, were unsuccessful (Materials and Methods, Sect 2.4 (ii), p75).

A further attempt to separate these bands was made by using a folate column (folate is an affinity ligand for XO); for further details of this column see Materials

Fig50 Standard curves showing the presence of IgG, IgA, and IgM anti-XO antibodies in a pooled serum , using two different preparations of XO, in the XO ELISA (Sect 5.4).

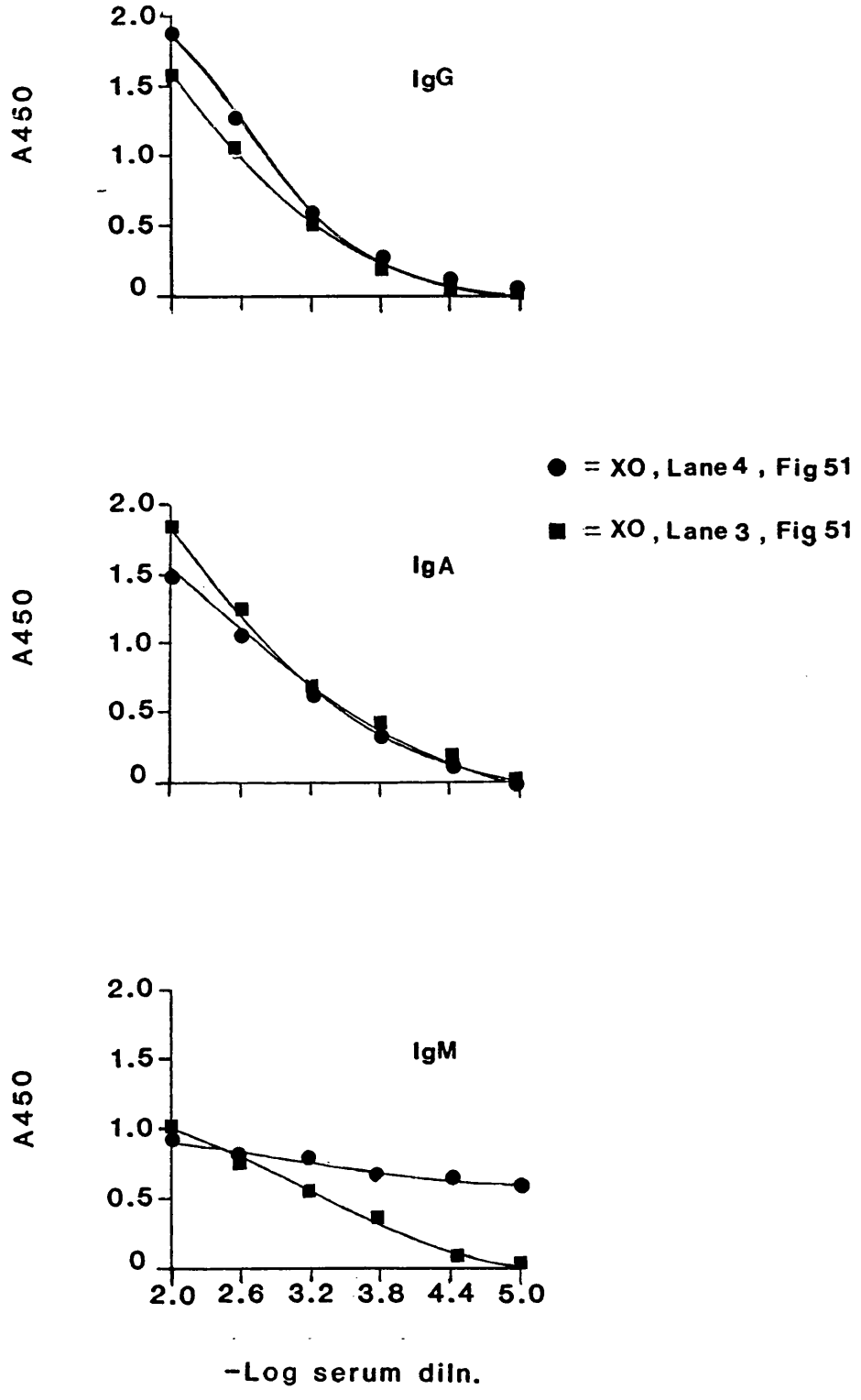
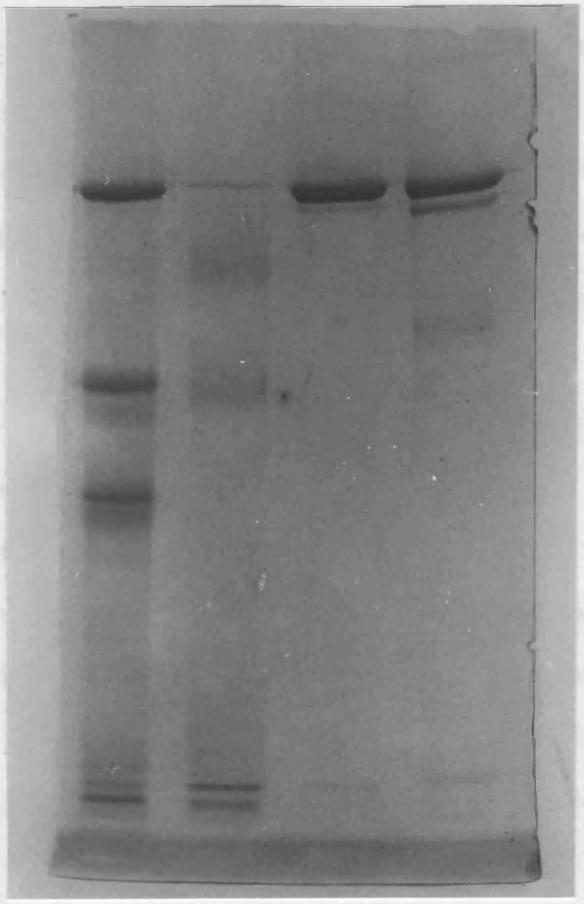


Fig 51 SDS-PAGE profile of two different preparations of XO , purified from cows' milk, by the method of Nakamura and Yamazaki, 1982.



L-R

1. BMFGM (75µg)
2. Non-binding extract of XO from BMFGM (20µg)
3. XO -no background (30µg)
4. XO -background (30µg)

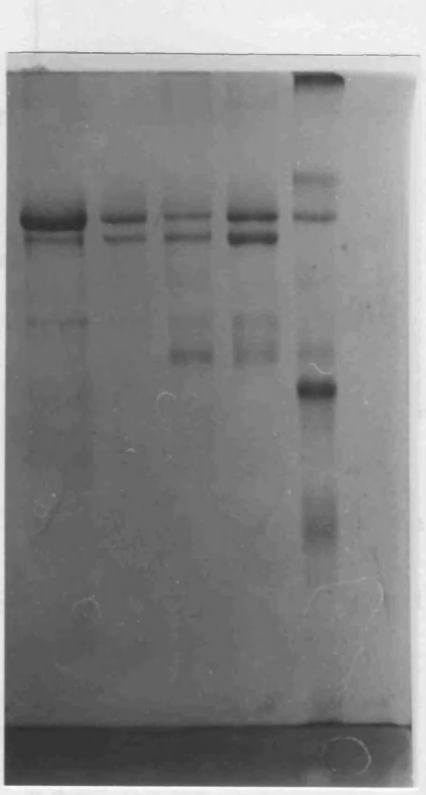
and Methods Sect 2.4 (iii), p76). The sample of XO (containing 150kD and 83kD polypeptides) was applied to the column (10ml) which was washed with Buffer A, until the column eluate A_{280} was < 0.005. Protein remaining on the column was affinity eluted by Buffer B. Of 25.4 ± 2.3 mg protein loaded onto the column, a colourless fraction of protein content 3.1 ± 0.7 mg passed through the column unbound, (no XO, or XD activity was recorded in this fraction), and an orange-brown fraction of protein content 20.4 ± 1.6 mg, of specific activity, 6.5 ± 0.3 IU/mg, was eluted by Buffer B (n=2).

Fig 52, p171, shows SDS-PAGE patterns of the fractions collected. It can be seen that the folate column effectively removes the 83kD band, which passes through unbound. When tested as antigens on an ELISA (10µg/ml carbonate buffer, the original sample and the unbound fraction (lanes 2, and 3, Fig 52, p171), gave high backgrounds, whereas the affinity eluted XO did not.

5.4 Design of an ELISA to detect anti-Xanthine Oxidase antibodies.

The ELISA protocol was similar to that of the BMFGM ELISA, described in Methods Sect. 3.3, p86. The main difference was that XO was suspended in carbonate coating buffer, in place of BMFGM as the antigen. All incubation, wash and staining steps were the same.
XO (prepared from the folate column) coating concentrations were optimised for the ELISA (as with BMFGM see p103).

Fig 52 SDS-PAGE profile of XO + contaminant applied to folate affinity column.



1. BMFGM (50µg)
2. XO applied to folate column (30µg)
3. Run through folate column (30µg)
4. XO specifically eluted from folate column (20µg)
5. XO after 2 days at 40°C (+ inhibitors)

Fig 53 Optimisation of XO coating concentrations for the ELISA.

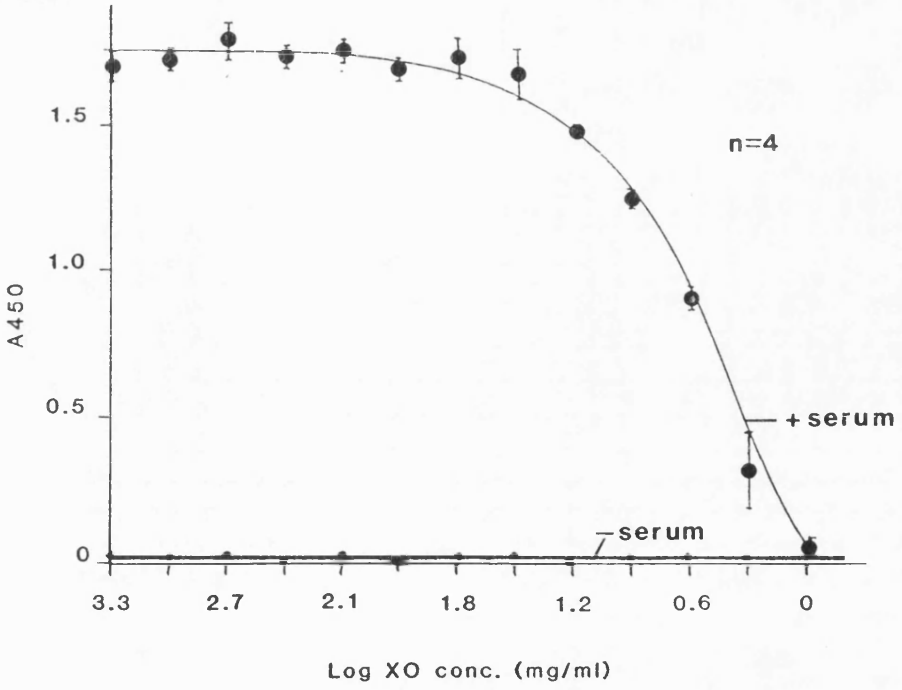
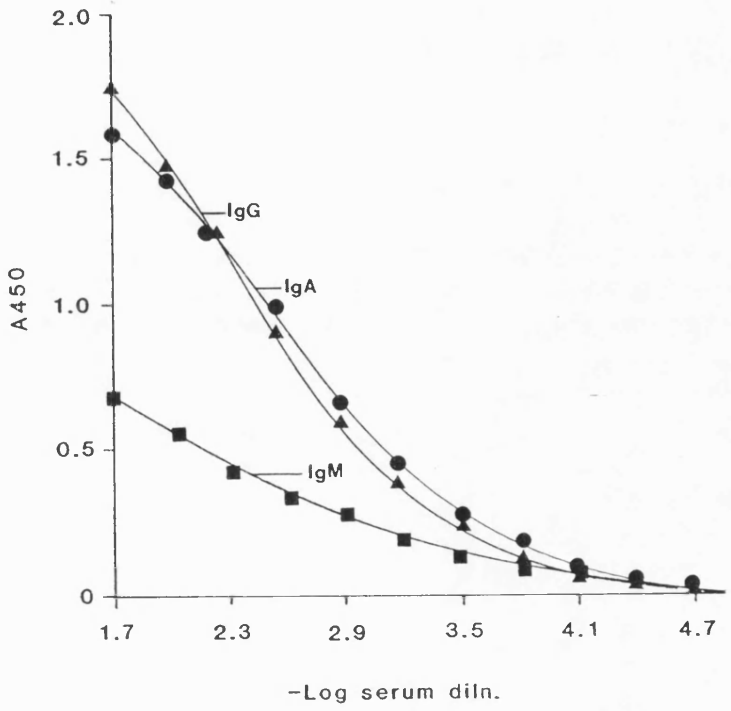


Fig 54 Standard curves produced from the detection of anti-IgG, anti-IgA and anti-IgM XO antibodies in a pooled human serum.



The results of this are shown in Fig 53, p172.
Fig 54, p172, shows the results of anti-XO IgG, IgA, and IgM detection in pooled sera, using goat-anti human IgG, IgA, and IgM (1:1000) conjugated HRP. The pooled sera which had been used to generate a standard curve in the BMFGM ELISA (Results Sect 1.3, p112), contained very little anti-XO IgA, or IgM. A second set of sera in which these antibodies were high were pooled and used for the standard curves shown in Fig 54. Anti-XO IgG samples were assayed at 1:200 as before, and anti-IgA-, and IgM were assayed at 1:100.
The final protocol is described in Materials and Methods Sect 3.3, p88.

5.5 Comparison of anti-XO antibody titres with anti-BMFGM antibody titres.

IgG, IgA, and IgM anti-XO antibody levels were assayed in 198 human serum samples and compared, in each sample with the corresponding anti-BMFGM antibody titre (see Fig 55, p174-177). Significant correlations were obtained for all antibody types.
[The Rs calculated when comparing anti-XO antibody titres with anti-BMFGM levels were 0.894 (IgG), 0.894 (IgA) and 0.599 (IgM). Values above 0.14 are necessary to show a significant correlation for p=0.05.]

5.6 A comparison of IgG, IgA, and IgM anti-XO antibodies in MI and Control samples.

The 100 MI samples, age and region matched with 100

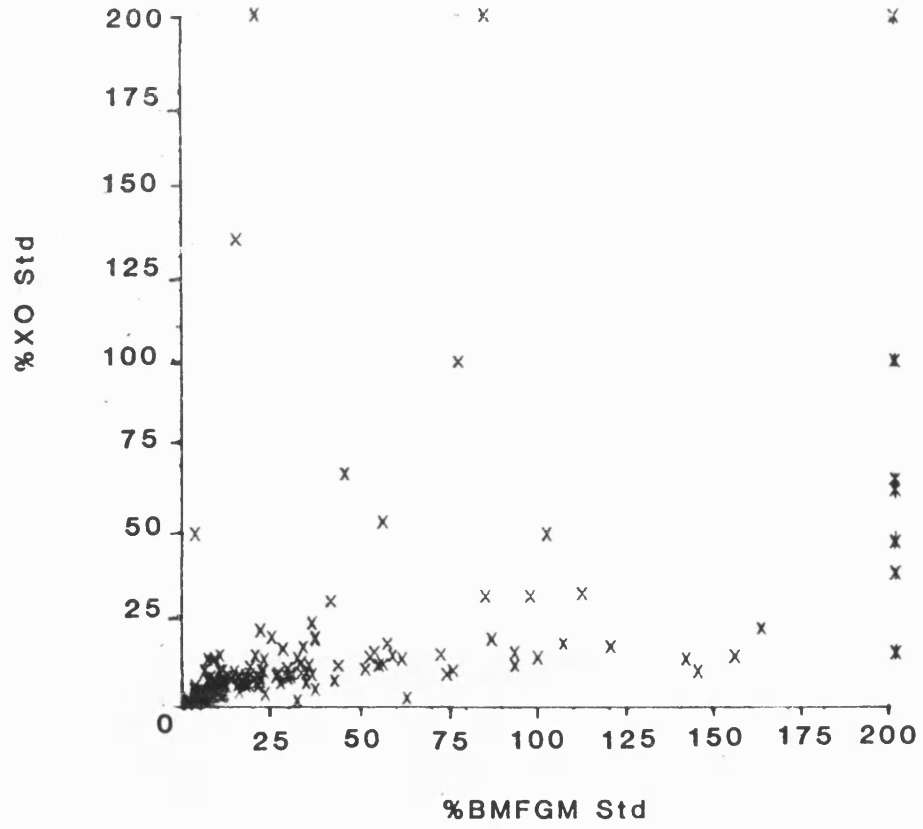
Fig 55 Comparisons of anti-BMFGM antibody titres with anti-XO titres in the same samples, measuring:-

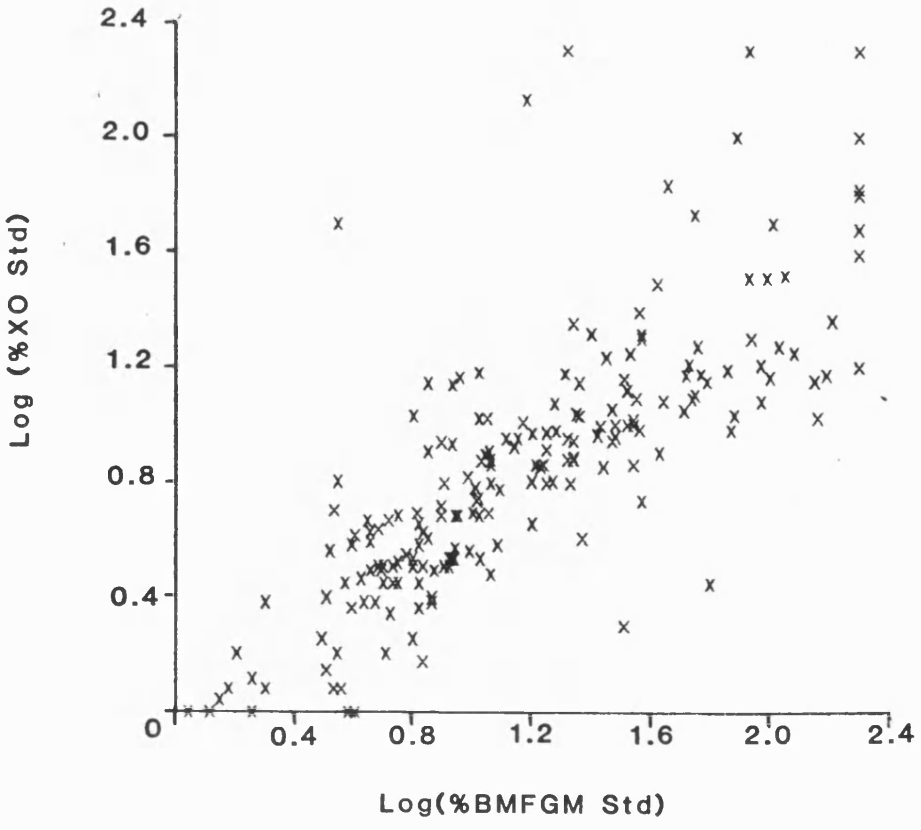
a) IgG

b) IgA

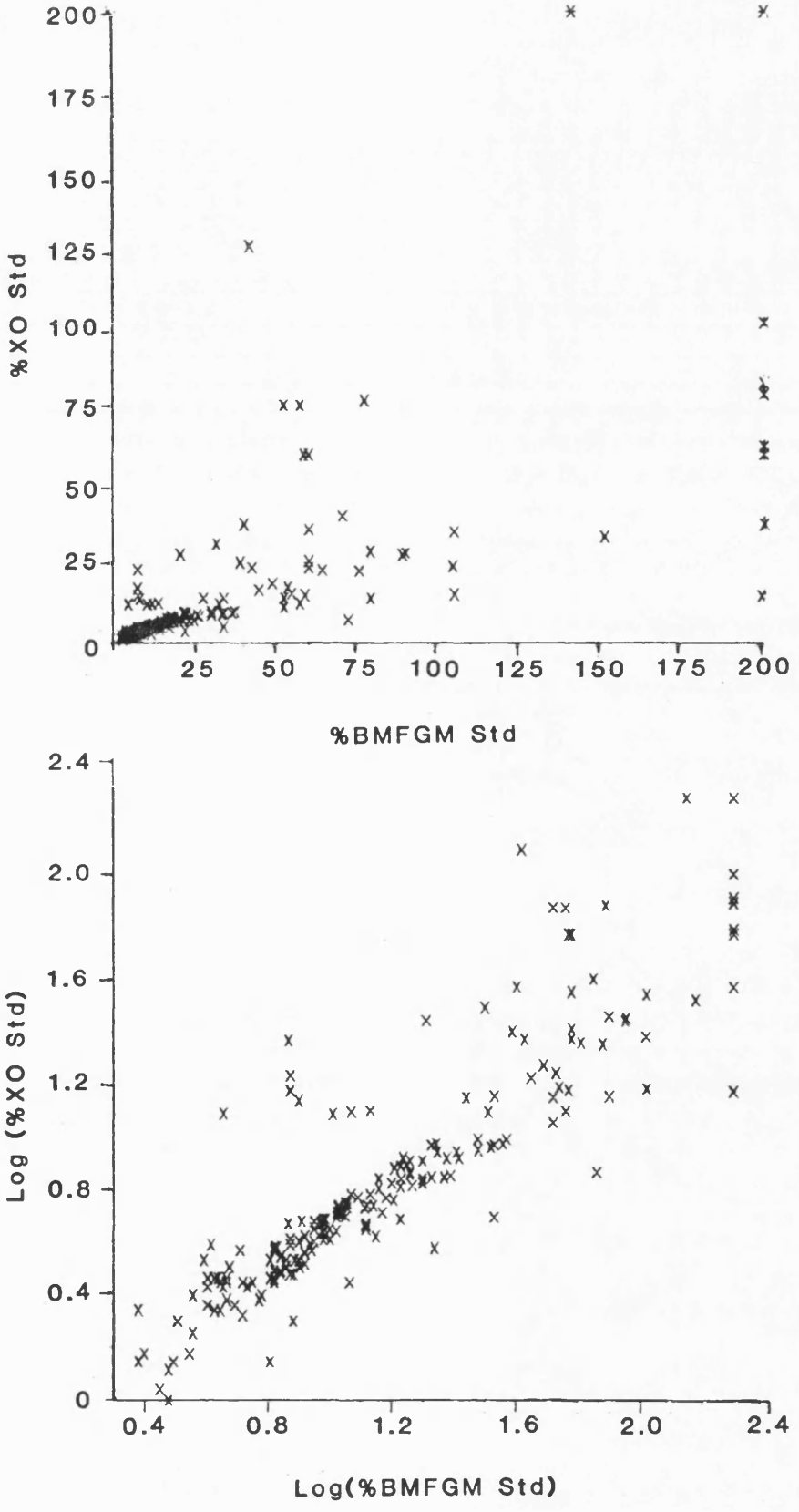
c) IgM levels.

a) IgG

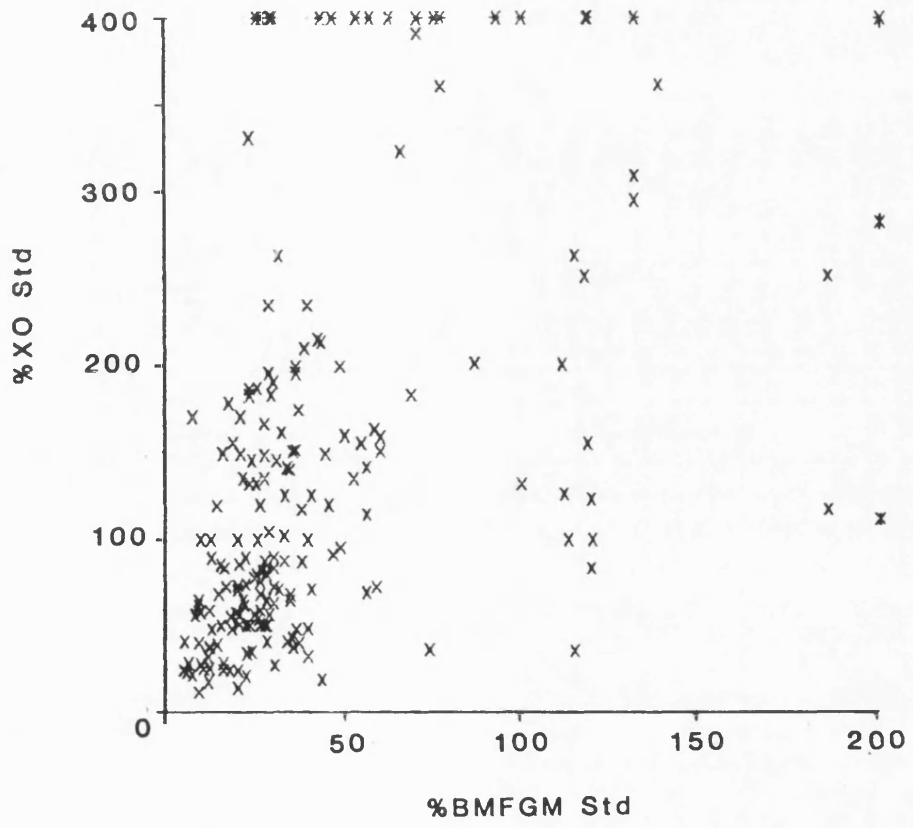

200
175
150
125
100
75
50
25
0
%XO Std
25
50
75
100
125
150
175
200
%BMFGM Std

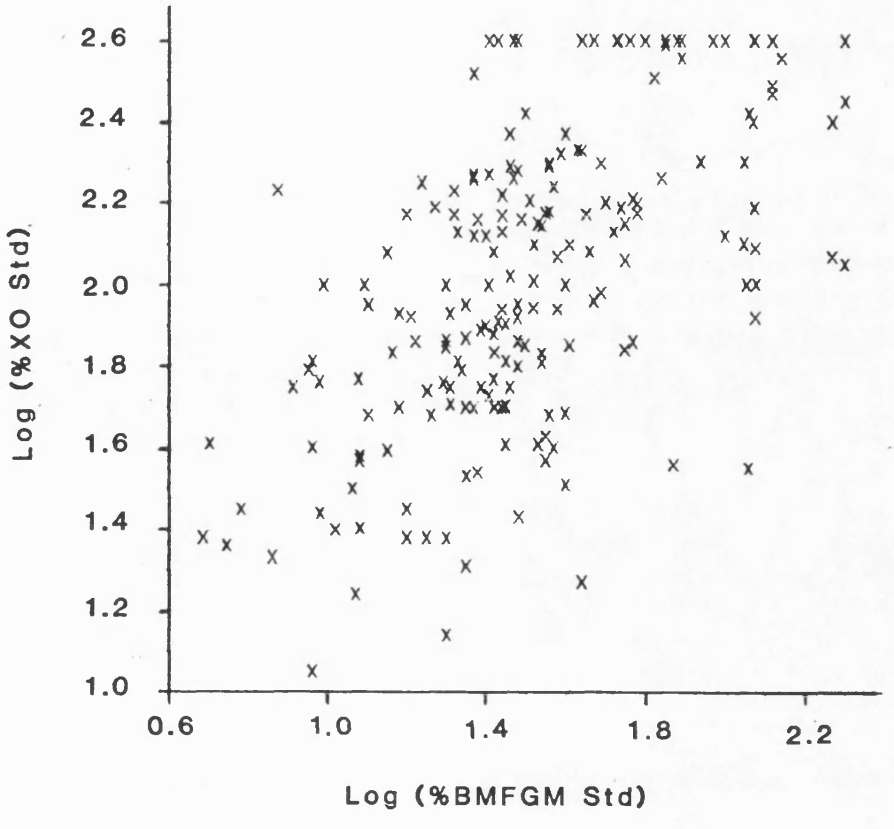

2.4
2.0
1.6
1.2
0.8
0.4
0
Log (%XO Std)
0.4
0.8
1.2
1.6
2.0
2.4
Log(%BMFGM Std)

b) IgA


200
175
150
125
100
75
50
25
0
%XO Std
25
50
75
100
125
150
175
200
%BMFGM Std
2.4
2.0
1.6
1.2
0.8
0.4
0
Log (%XO Std)
0.4
0.8
1.2
1.6
2.0
2.4
Log(%BMFGM Std)

c) IgM


400
300
200
100
0
%XO Std
50
100
150
200
%BMFGM Std


2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
Log (%XO Std)
0.6
1.0
1.4
1.8
2.2
Log (%BMFGM Std)

controls, used to compare anti-BMFGM antibody levels (Results Sect 4.2, p144) were assayed for anti-XO antibodies using the ELISA system described in Results Sect 5.4. Fig 56, p180, shows the results of these comparisons; only IgM anti-XO antibodies are significantly raised in MI patients.

[For IgG anti-XO antibodies the point above which the MI samples and controls differ most is at 20-30% of the Std (11% of MI, and 9% of control samples had values greater than this). A K-S value of 0.02 is well below the theoretical value of 0.192, necessary to show a significant difference between the two populations at p=0.05.

For IgA, the region of maximum difference was above 0-5.0% of the Std (39% of MI and 50% of control samples had values greater than this). A K-S value of 0.11 calculated for this difference was again below the value of 0.192, necessary to show a significant difference.

For IgM, there were two places above which the two populations of MI and controls were equally different, these were at 50-100% of the Std, and 100-150% of the Std (50% of MI and 30% of contol samples had values greater than 150% of the Std; 69% MI and 49% control were above 100% Std). A K-S value of 0.2, was greater than the necessary value of 0.192 to show a significant difference between the two populations at p=0.05]

A second series of MI patients whose sera was available 1-2 days after infarction was available from the Royal United Hospital (Bath, Avon, U.K.). Anti XO

Fig **56** Distribution of XO antibodies in 100 MI patients (age and region matched) with 100 controls, measuring:-

a) anti-XO IgG

b) anti-XO IgA

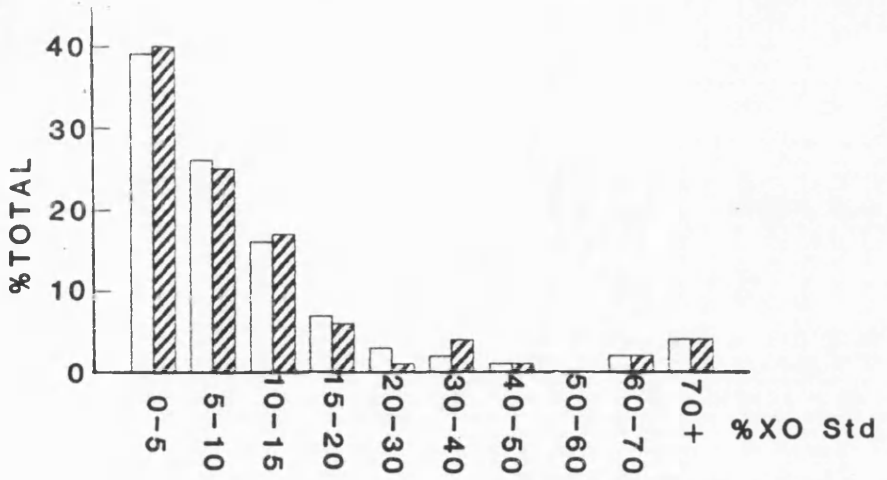
c) anti-XO IgM antibodies.

Key:-

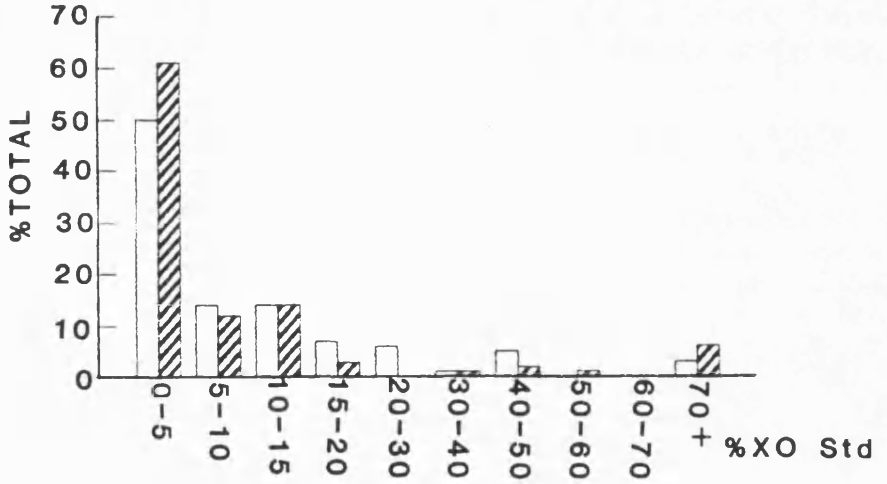
☐ = Control

▨ = MI

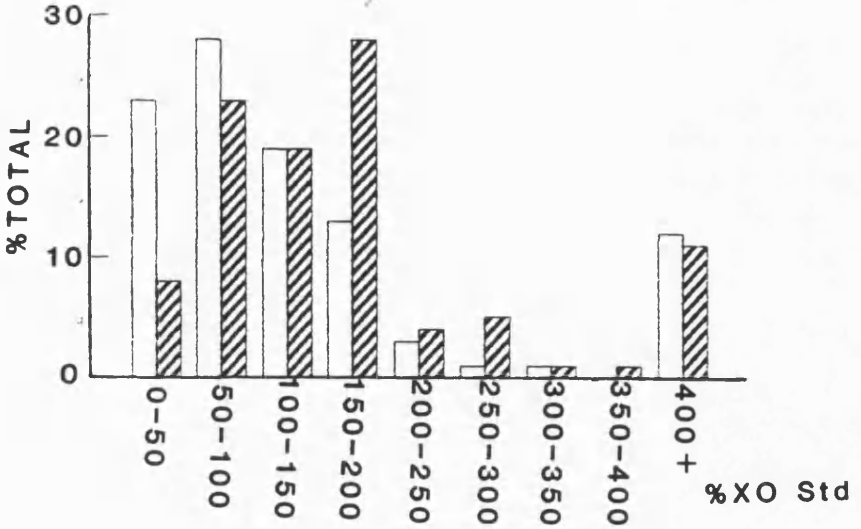
a)


%TOTAL
40
30
20
10
0
0-5
5-10
10-15
15-20
20-30
30-40
40-50
50-60
60-70
70+
%XO Std

b)


%TOTAL
70
60
50
40
30
20
10
0
0-5
5-10
10-15
15-20
20-30
30-40
40-50
50-60
60-70
70+
%XO Std

c)


%TOTAL
30
20
10
0
0-50
50-100
100-150
150-200
200-250
250-300
300-350
350-400
400+
%XO Std

levels were similarly measured in these and compared to age matched hospitalised (same hospital, Bath RUH) patients who had no known history of heart disease. The results of this study are shown in Fig 57, p183. Again, IgM anti-XO antibodies are raised in MI patients.

[For IgG a maximum difference between the two populations occurs above 0-5.0% of the Std (48% of MI, and 61% of control are greater than 5.0% of the Std). A K-S value of 0.136 was below that necessary for significance, 0.274 for p=0.05.

For IgA, the maximum difference occurred at 0-5.0% of Std also (16% of MI, and 30% of control were above this). A K-S value of 0.243 was again below 0.274.

For IgM, the greatest difference occurred above 100-150% of the Std (54% of MI and 11% of control samples had values greater than this). A K-S value calculated at 0.42 was much greater than that necessary for significance at p=0.05, 0.274.

Fig 57 Distribution of XO antibodies in 56 MI patients (1-2 days after infarction), with 44 hospital controls, measuring:-

a) anti-XO IgG

b) anti-XO IgA

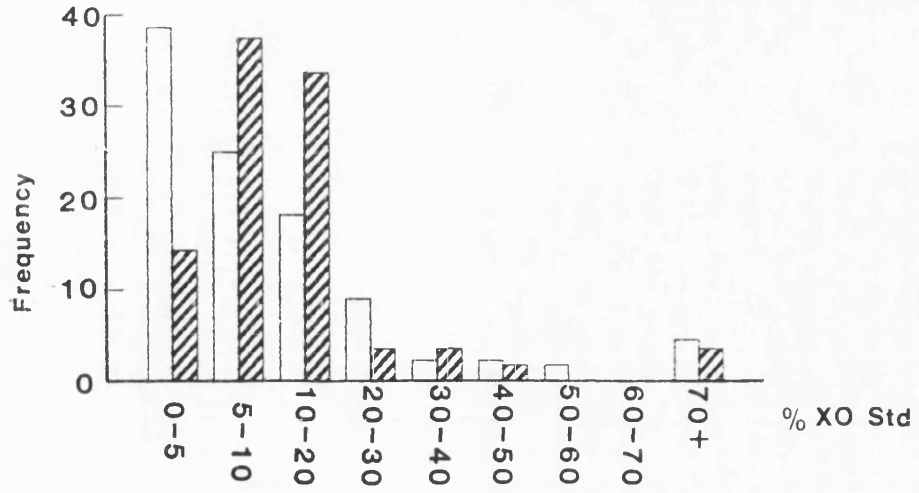
c) anti-XO IgM antibodies.

Key:-

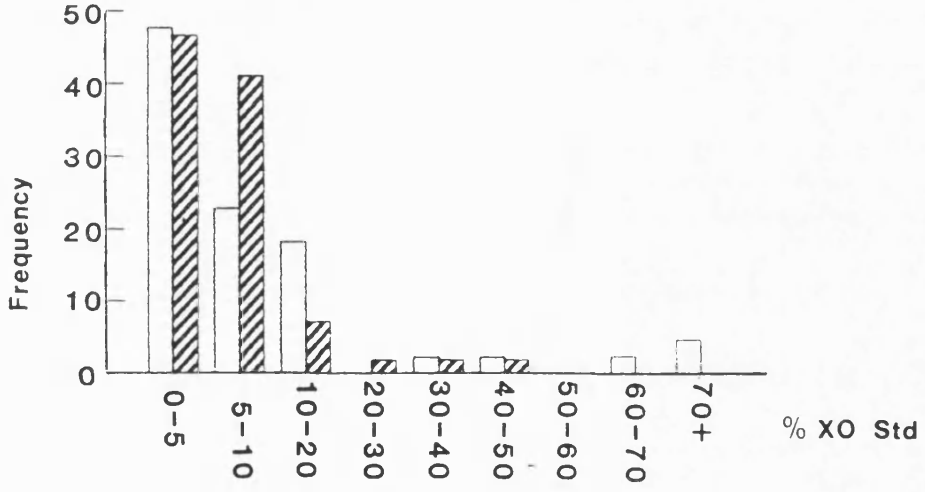
☐ = Control

▨ = MI

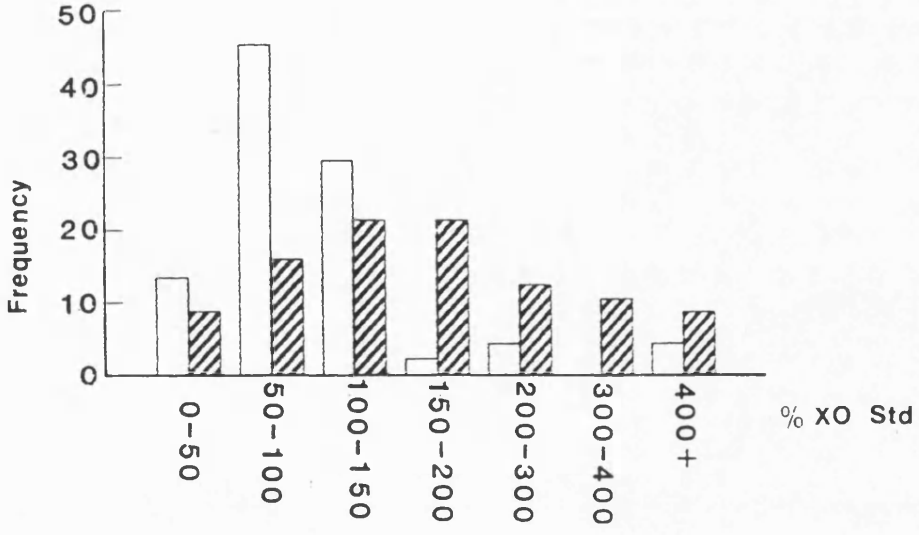
a)


40
30
20
10
0
Frequency
0-5
5-10
10-20
20-30
30-40
40-50
50-60
60-70
70+
% XO Std

b)


50
40
30
20
10
0
Frequency
0-5
5-10
10-20
20-30
30-40
40-50
50-60
60-70
70+
% XO Std

c)


50
40
30
20
10
0
Frequency
0-50
50-100
100-150
150-200
200-300
300-400
400+
% XO Std

6.0 IMMUNOLOGICAL CROSS-REACTIVITY OF BMFGM WITH PLATELET MEMBRANE.

6.1 Design of an ELISA to measure anti-Platelet membrane antibodies.

a) The method of Horai et al (1981) was first investigated.

A platelet suspension (50µl, 10^4/µl), was added to microtitre wells and the trays were centrifuged (5min, 200g av.). Formaldehyde (50µl, 2% in PBS) was added to each well and left for 1h at room temperature. The wells were then washed (4 x 10 min) with PBS/Tween (0.05%). Serum was then added to each well (appropriately diluted in washing buffer). After incubation (1h, 37°C), wells were washed as before, then incubated (45min, 37°C) with goat anti-human IgG-HRP conjugate (as in Results Sect 1.1, p102, 1:1000 in PBS/Tween 20, 0.05%). Wells were washed again and stained as described in the BMFGM ELISA, Materials and Methods sect. 3.3 (i), p87.

Table 23, p187, shows the results obtained when a 1:50 dilution of serum was coated onto 75 wells. There were essentially two problems 1. A wide variation in replicates of the same sample, and 2. A high background reading in the blank well.

Poly-l-lysine was used to pre-coat microtitre wells (100µl/well,50µg/ml PBS, 1h at 37°C), before coating with platelets and calcium was included in the buffer (10mM in TBS, not PBS) to try to improve the binding of

the platelets to the microtitre plates. But, as is obvious from Table 23, p187, no significant improvements were made. Table 24, p187, shows the results of addition of various blocking agents to the buffer used in the ELISA. Again, no significant reduction was made in the background levels.

To try to eliminate background absorption, presumably arising from platelet bound IgG, the platelet monolayers were incubated and washed with buffer for various times, at 37°C. Table 25, p187 shows that, again, this had no effect in reducing the background absorption.

b) The method according to Winiarski and Ekelund,(1986) was followed.

Platelet membrane, prepared by sonication (100W, 60s), of a washed platelet suspension, Materials and Methods Sect 2.3, p74, was suspended in PBS (50µg membrane protein/ml) and then added to microtitre wells (100µl/well). Formaldehyde (100µl, 2% in PBS) was added to each well and allowed to stand for 1h at 25°C. Plates were washed (5 x 5 min) with PBS/Tween 20, 0.05%. Serum was added to each well (1:100 diluted in PBS/Tween, 4%BSA). The plates were incubated (1h, 37°C) and washed as before. Conjugate (as, Sect 6.1.a, p184) was added to each well (100µl, 1:1000 PBS/Tween). After incubation (45min, 37°C) wells were washed, as above, stained and read for the ELISA, as in Materials and Methods sect. 3.3, p87.

Table 23 Effect of different coating conditions of platelet monolayers on the reproducibility of the ELISA.

Table 24 Effect of different blocking conditions on the platelet ELISA background (i.e. wells with no serum). PBS/Tween with asein (1% for washing, and 0.1% when incubating with serum and conjugate), Gelatin (0.1% in all buffers), Normal goat serum (NGS), and Normal rabbit serum (NRS), (5%, with incubation buffer).

Table 25 Effect of pre-incubation of platelet monolayers with buffer, to remove surface bound IgG, on absorbance of wells with no sera.

Coating of microtitre wells	- serum	+ serum
Platelet Monolayer (PM)	0.210 ± 0.180	0.361 ± 0.284
PM + poly-L-lysine	0.192 ± 0.089	0.354 ± 0.216
PM +poly-L-lysine + Ca^{2+}	0.280 ± 0.120	0.326 ± 0.209

n = 5

Blocking agent	A_{450}
CASEIN (1%)	0.150 ± 0.101
NGS (5%)	0.175 ± 0.162
NRS (5%)	0.206 ± 0.189
BSA (1%)	0.183 ± 0.146

n = 3

Incubation time of platelet monolayer with buffer (min)	A_{450}
1 x 30	0.193 ± 0.120
3 x 30	0.184 ± 0.131
10 x 30	0.190 ± 0.096

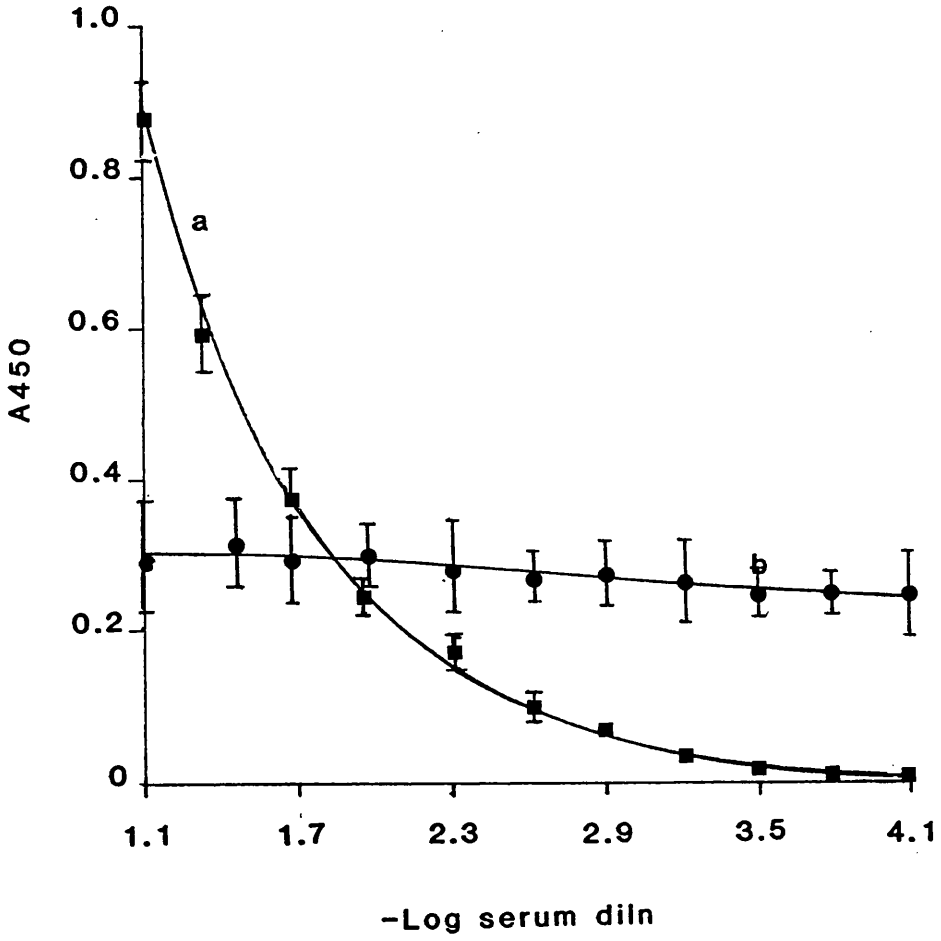
n = 3

Fig 58, p189, shows a standard curve obtained by using the BMFGM standard serum. This is compared to the "standard curve" generated by the method of Horai et al. Table 26, p190, shows a range of titres produced from 12 different sera of known anti-BMFGM titre. As can be seen, the anti-platelet titres range between 11-26% of the Std, unlike the BMFGM titres which range from 2.3-102% of the Std. Further attempts were accordingly made to try to improve the ELISA, by eliminating some of what was probably non-specific binding of the serum to platelet membrane. A number of parameters were further investigated. These were:-

i Platelet membrane preparation

The method of membrane preparation used by Winiarski and Ekelund (1986) involves sonication. However, it was noticed that certain batches of membrane produced a very high background with no serum. Fig 59, p191, shows that this background varies with the time of sonication, leading to inconsistencies and probably involving some structural damage to the membrane. Fig 60, p191, shows a change in the SDS-PAGE profile of platelet membrane after different times of sonication, most noticeably a mass of proteinaceous material appears around 60kD, after longer sonication times. An alternative method of membrane preparation was examined (Barber and Jamieson,1970, Materials and Methods Sect 2.2, p72). This preparation involves loading platelets internally with glycerol, then subjecting them to osmotic lysis.

Fig 58 Standard curve obtained by using the BMFGM Std serum on a platelet ELISA performed; a) according to the method of Winiarski and Ekelund (1986), and b) according to Horai et al, 1981.



Absorbance values are ± Standard Error (n = 4).

Table 26 A comparison of BMFGM titres with Platelet membrane titres, using the platelet membrane ELISA of Winiarski and Ekelund (1986).

Serum sample	Antigen	
	BMFGM (% Std)	PLATELET MEMBRANE (% Std)
1	2.3 ± 1.2	19.1 ± 3.2
2	4.1 ± 0.6	16.2 ± 1.3
3	6.5 ± 1.7	11.2 ± 2.1
4	8.1 ± 1.9	20.4 ± 5.2
5	12.8 ± 3.4	20.4 ± 6.3
6	15.8 ± 1.6	26.3 ± 4.7
7	30.7 ± 5.5	19.0 ± 3.9
8	42.4 ± 8.1	24.6 ± 5.9
9	75.5 ± 9.7	26.3 ± 4.8
10	79.1 ±10.6	19.1 ± 2.2
11	100.0 ± 9.3	26.0 ± 4.3
12	102.5 ±14.6	24.6 ± 6.2

n = 3

Fig 59 Effects of different sonication times (for the preparation of platelet membrane) on the background absorbances of the platelet membrane ELISA i.e. absorbances produced in microtitre wells with no serum.

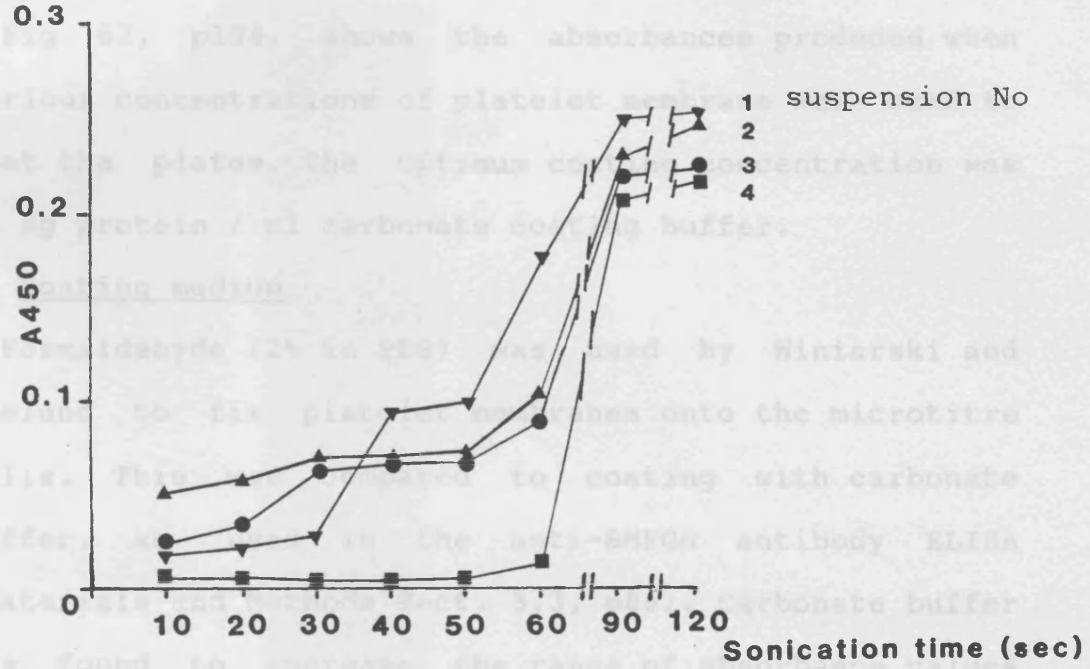
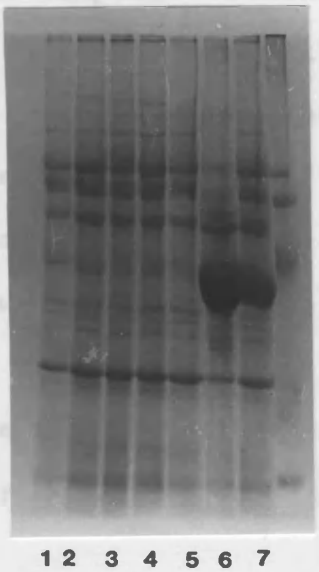


Fig 60 SDS-PAGE profiles of a platelet membrane (suspension 2, of above), following sonication for different time intervals.



Key:-

Lane	Sonication time (sec.)
1	20
2	30
3	40
4	50
5	60
6	90
7	120

Platelet membranes are then recovered on a sucrose density gradient. SDS-PAGE profiles (Fig 61, p193) of membrane preparations from the two techniques, are very similar. However, membrane prepared without sonication gives lower background levels.

Fig 62, p194, shows the absorbances produced when various concentrations of platelet membrane were used to coat the plates. The optimum coating concentration was 10 µg protein / ml carbonate coating buffer.

ii Coating medium

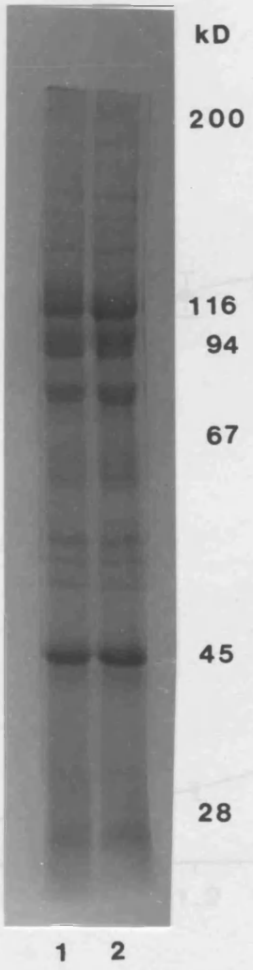
Formaldehyde (2% in PBS) was used by Winiarski and Ekelund to fix platelet membranes onto the microtitre wells. This was compared to coating with carbonate buffer, as used in the anti-BMFGM antibody ELISA (Materials and Methods Sect. 3.3, p86). Carbonate buffer was found to increase the range of absorbance values (Fig 63, p195), and was accordingly used subsequently.

iii Blocking agents

Table 27, p196, compares the antibody titres of 10 serum samples obtained in the presence and absence of NRS (5%). Its effect varies for each serum suggesting that it has a specific effect in reducing antibody titres, e.g. the titre of serum sample 4 is dramatically reduced, whereas that of sample 1 is not. NRS was included in all subsequent ELISAs.

The finalised Platelet Membrane ELISA is described in Materials and Methods Sect 3.3 (iii), p88.

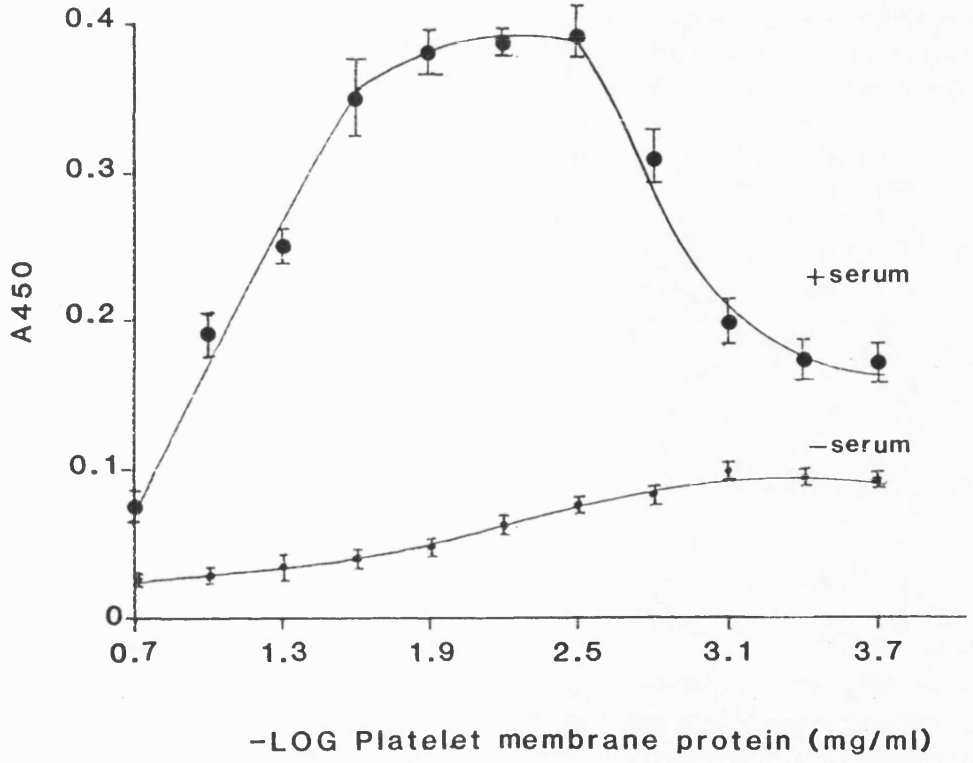
Fig 61 SDS-PAGE profile of two different platelet membrane preparations, which react differently as antigens on a platelet membrane ELISA.



Lane 1 - Winiarski and Ekelund, 1986.

Lane 2 - Barber and Jamieson, 1970.

Fig 62 Optimisation of platelet membrane coating concentration for the ELISA.



Absorbance values are ± Standard Error (n = 4).

Fig 63 Optimisation of membrane coating conditions for the platelet ELISA, using a) Formaldehyde, and b) carbonate coating buffer.

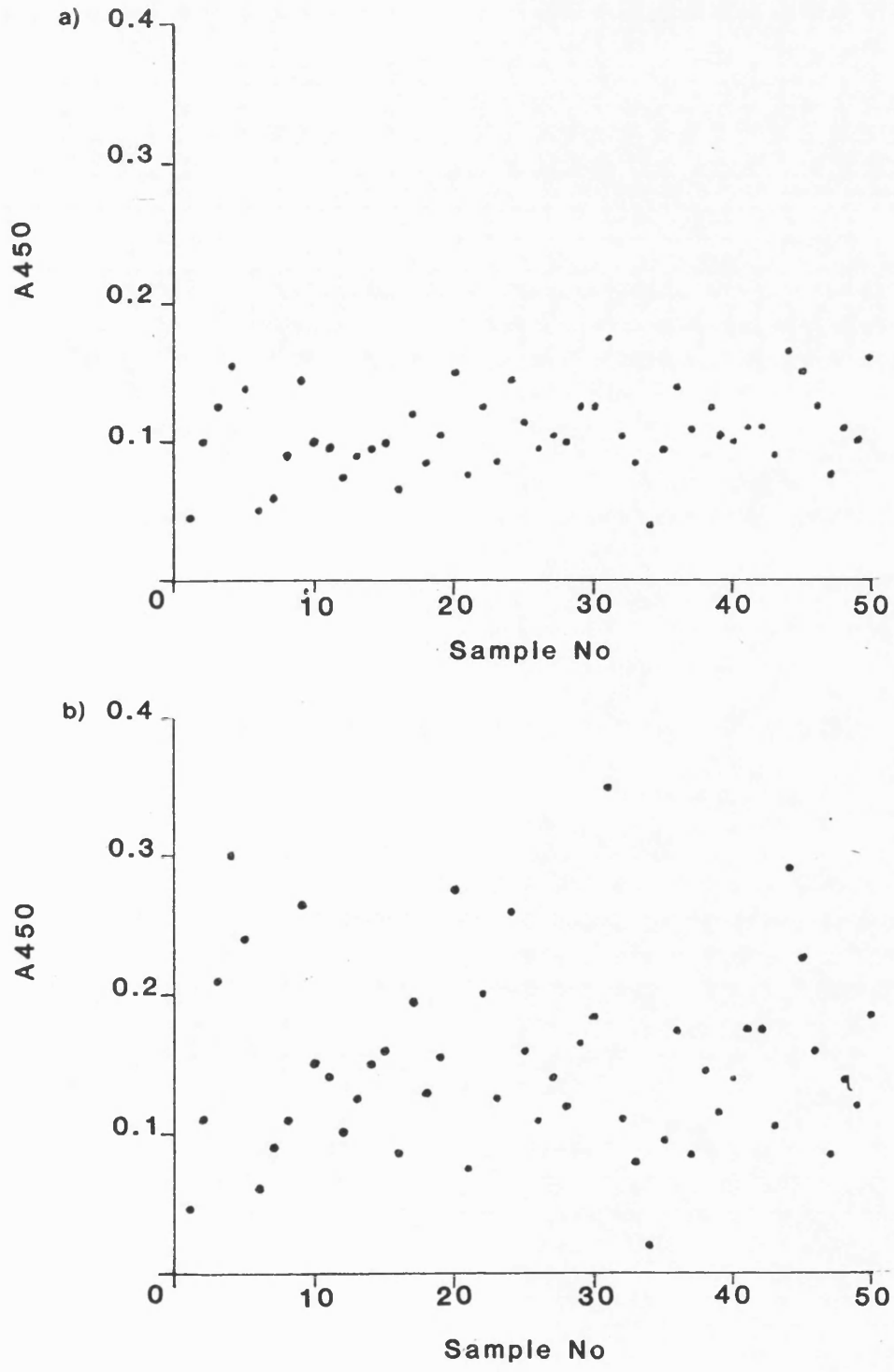


Table 27 The effect of including NRS in the platelet ELISA.

Serum Sample	% Std	%Std + NRS
1	30.1 ± 4.6	29.2 ± 3.1
2	25.2 ± 2.3	10.4 ± 1.2
3	18.3 ± 2.3	15.5 ± 1.9
4	16.2 ± 2.4	8.3 ± 1.6
5	15.5 ± 1.9	14.1 ± 0.8
6	13.9 ± 3.2	14.4 ± 1.2
7	12.4 ± 1.4	12.1 ± 2.6
8	10.9 ± 2.1	6.3 ± 1.4
9	11.4 ± 1.3	10.1 ± 0.4
10	8.2 ± 1.4	7.1 ± 1.4

6.2 Comparison of anti-BMFGM antibody titres with anti-platelet membrane titres.

The ELISA developed in Sect 6.1.b was used to compare anti-Platelet membrane antibody titres with anti-BMFGM antibody titres.

6.2.1 Comparative assays

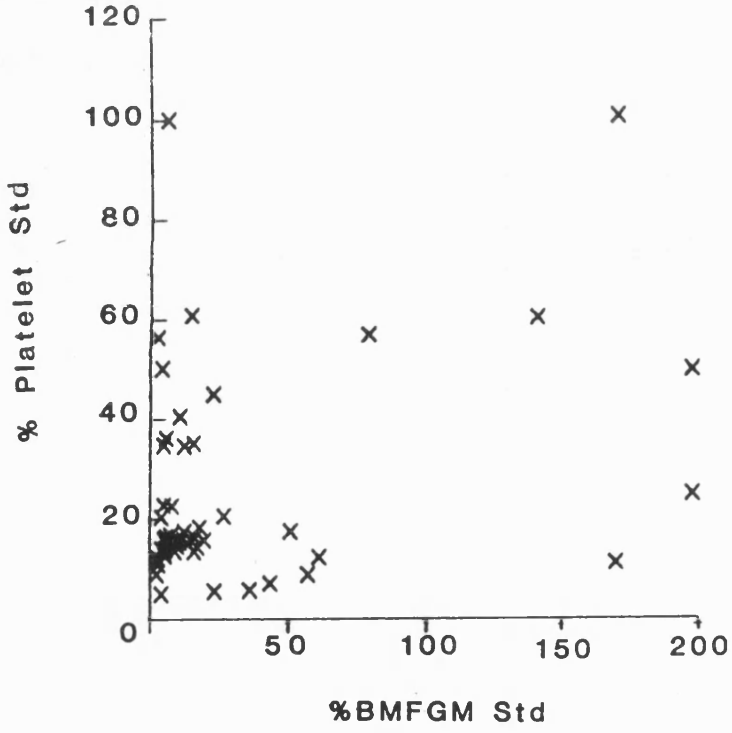
Fig 64, p198, shows a comparison of anti-BMFGM titres with anti-Platelet membrane titres, measured in 50 serum samples. The average titres (of three separate measurements) are shown. The rank correlation coefficient (Rs = 0.088) calculated is below that necessary to show a significant correlation (Rs = 0.273) for p = 0.05.

Table 28, p199, shows the results of assaying affinity-purified anti-BMFGM antibodies for anti-platelet membrane activity. No absorbance was shown by any sample. In contrast, IgG purified from rabbit anti-platelet membrane serum (Materials and Methods Sect. 2.5, p77), did produce a high titre.

Control sera from healthy donors (as used in the BMFGM ELISA) were compared with Immune Thrombocytopaenia (ITP) sera, using the anti-platelet membrane ELISA (Fig 65, p200).

A maximum difference was obtained above 15-20% of the Std (34% of the controls and 56% of ITP sera had titres above this.) A K-S value calculated for this difference of 0.2 was below that necessary (0.289 for p=0.05) to show a significant difference.

Fig 64 Correlation of anti-BMFGM antibody titres with anti-Platelet membrane titres.



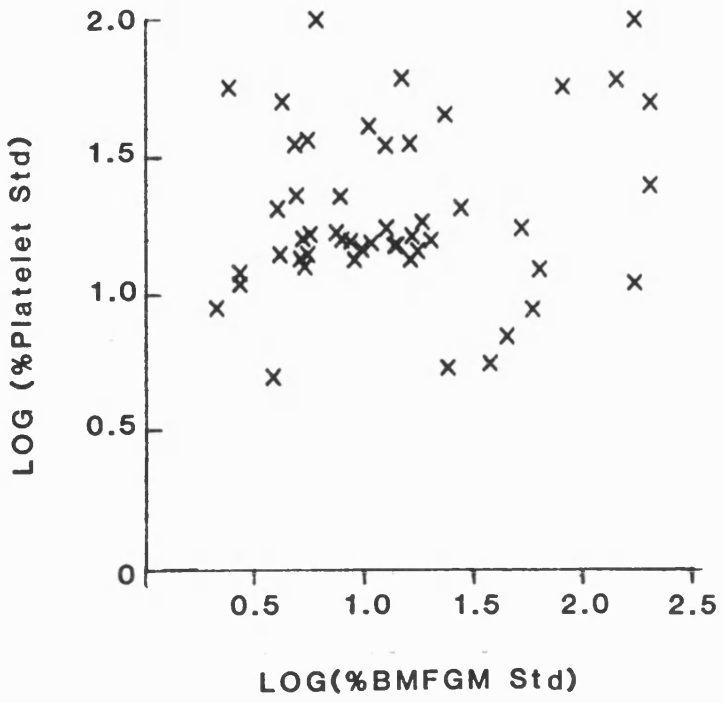
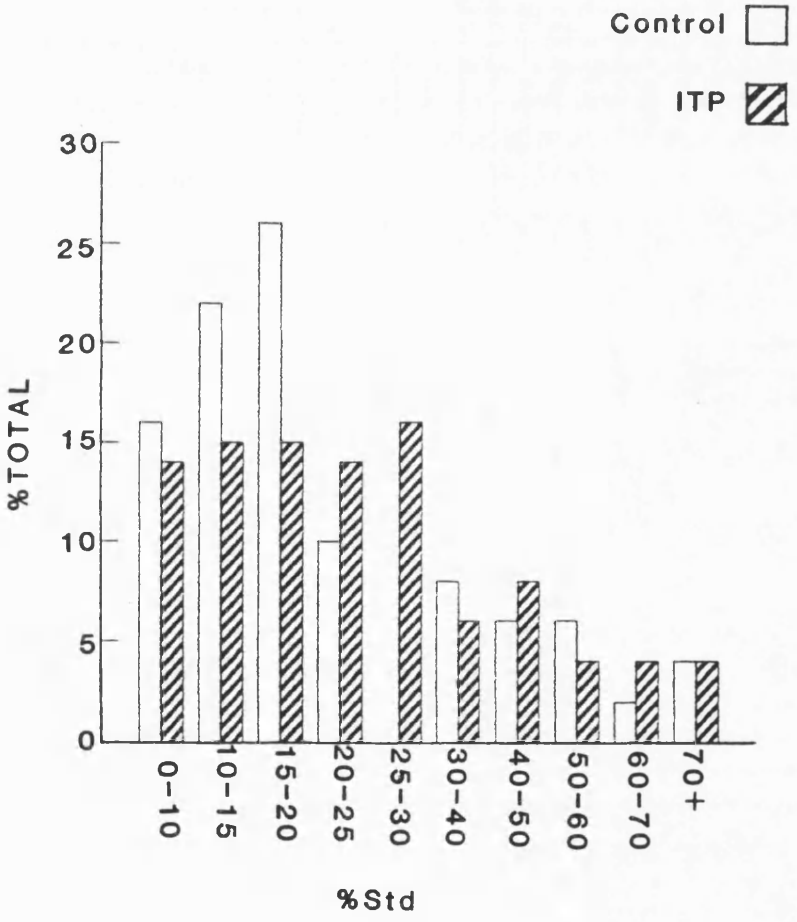


Table 28 Affinity purified anti-BMFGM antibodies assayed for anti-platelet membrane activity.

Sample No	BMFGM Titre [% Std]	Pl.Memb. Titre [% Std]
1	123.4 ± 12.2	0
2	100.3 ± 9.6	0
3	89.6 ± 7.1	0
4	82.5 ± 8.0	0
5	46.9 ± 5.8	0
6	32.7 ± 4.3	0
7	20.4 ± 5.1	0
8	10.6 ± 4.3	0
9	5.7 ± 2.5	0
10	2.2 ± 1.6	0
Rabbit anti-Platelet Membrane IgG.	0 ± 0	120.6 ± 15.2

Fig 65 Comparison of ITP sera with normal sera using the anti- platelet membrane antibody ELISA.



6.2.2 Competitive ELISAs

Platelets were used as competitors in the BMFGM ELISA as follows. Platelets were either used directly, as PRP, or washed as described in Materials and Methods Sect 2.3, p74, and those pre-blocked were incubated in PBS/Tween, NRS(5%) (1h, 37°C). Platelet suspensions (0.125-64 x 10^6/ml) were mixed with an appropriate amount of diluted serum (1:100), in order to give the required final concentrations of PBS/Tween and serum, and added to microtitre wells (100µl/well). After incubation, plates were washed, and the ELISA was followed according to Materials and Methods Sect 3.3, p88.

Table 29, p202, shows the effects of using different concentrations of platelets as competitors using a sample of pooled human serum by BMFGM ELISA for anti-BMFGM antibodies. Far from inhibiting the assay as expected, all additions led to an apparent increase in anti-BMFGM levels measured.

An alternative means of assaying platelet incubated sera was examined. Platelets (blocked and non-blocked as above) were incubated with a pooled serum, (undiluted) (1h, 37°C), to give final concentrations of 0.15-5.00 x 10^6/ml of serum. Platelets were then separated from the serum by centrifugation (10min, 12,000g av) and the remaining serum was assayed using the BMFGM ELISA, Materials and Methods Sect. 3.3, p86, and the Platelet Membrane ELISA, Materials and Methods, Sect 3.3, p88.

Incubation of the serum with washed platelets caused up to 30% inhibition of ELISA for anti-BMFGM antibodies

Table 29 Effect on anti-BMFGM antibody titres of platelets (Platelet rich plasma (PRP),washed, washed and blocked) used as competitors in the BMFGM ELISA.

	Platelet type		
Platelet conc. x 10^6/ml	PRP	Washed	Washed + blocked
64	190 ± 10	142 ± 20	161 ± 21
32	192 ± 6	150 ± 18	158 ± 18
16	190 ± 10	136 ± 25	169 ± 4
8	180 ± 15	147 ± 8	174 ± 36
4	195 ± 25	120 ± 15	180 ± 24
2	170 ± 30	148 ± 21	161 ± 17
1	200 ± 27	119 ± 30	193 ± 31
0.5	198 ± 17	120 ± 25	158 ± 3
0.25	194 ± 36	143 ± 8	169 ± 16
0.125	184 ± 9	143 ± 11	149 ± 15

(n = 3)

Titres are expressed as a % of the BMFGM Std.

Original serum titre = 105% of the Std.

Fig 66 Levels of anti-BMFGM antibodies in sera pre-absorbed with blocked and non-blocked platelets.

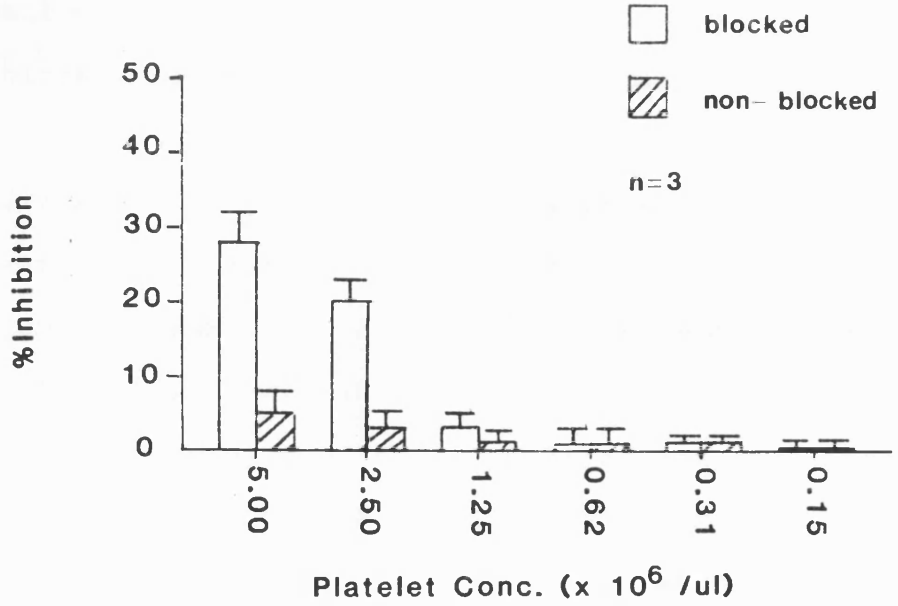
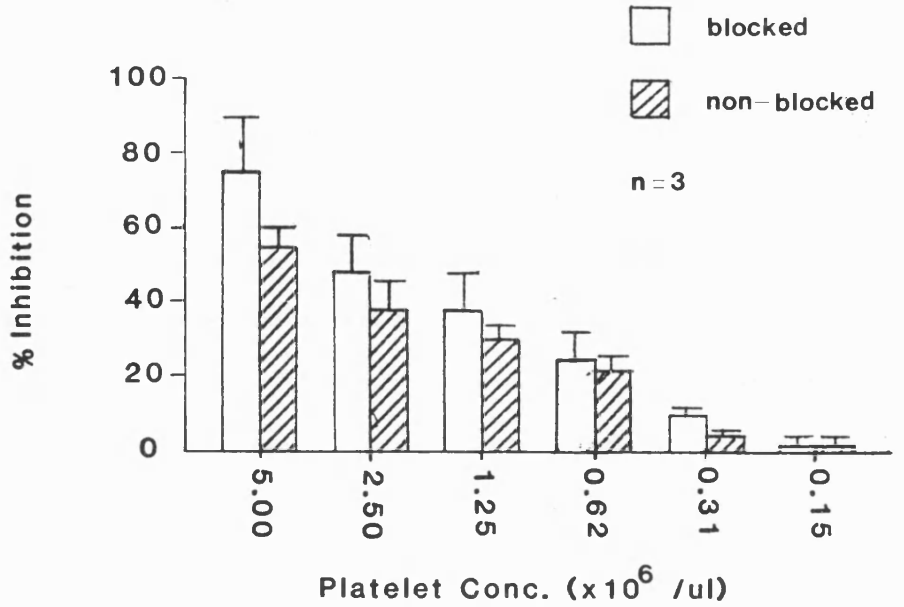


Fig 67 Levels of anti-platelet membrane antibodies in sera pre-absorbed with blocked and non-blocked platelets.



(see Fig 66, p203). Pre-incubation of the platelets with a blocking agent removed most of this inhibition. However as expected, ELISA for anti-human platelet antibody could be inhibited by either blocked or unblocked platelets see Fig 67, p203.

6.2.3 Assay of experimental anti-sera

Various rabbit anti-sera raised against BMFGM,(RABMFGM), XO (RAXO), Platelet Membrane (RAPM) and BSA (RABSA), (Materials and Methods Sect 2.8, p81) were assayed by using the following ELISA systems.

i Using the BMFGM ELISA (Materials and Methods Sect 3.3 (i), p86), the above anti-sera were assayed, along with their corresponding pre-immune sera (from dilutions of 1:50-1:2000), using a commercially available goat anti-rabbit-Polyvalent antibody-HRP conjugate.

Fig 68a, p206, shows that both RABMFGM and RAXO sera gave strong responses, whereas RABSA showed a moderate response and RAPM showed no response.

ii Using the ELISA to detect anti-XO antibodies (Materials and Methods Sect 3.3 (ii), p88), the antisera used in i above, at the same dilutions and using the same conjugate were assayed.

Fig 68b, p206, shows that RAXO and RABMFGM both gave strong responses, whereas RABSA and RAPM did not.

iii Using the ELISA to detect anti-platelet membrane antibodies (Materials and Methods Sect 3.3 (iii), p88), the anti-sera used in i and ii were similarly assayed.

Fig 68c, p206, shows that RAPM gave a strong response,

Fig 68 Assays of different rabbit anti-sera raised against; BMFGM, BXO, platelet membrane (PM), and BSA.

Key:-

■ = rabbit anti-BMFGM antisera

● = '' anti-XO ''

▼ = '' anti-PM ''

▲ = '' anti-BSA ''

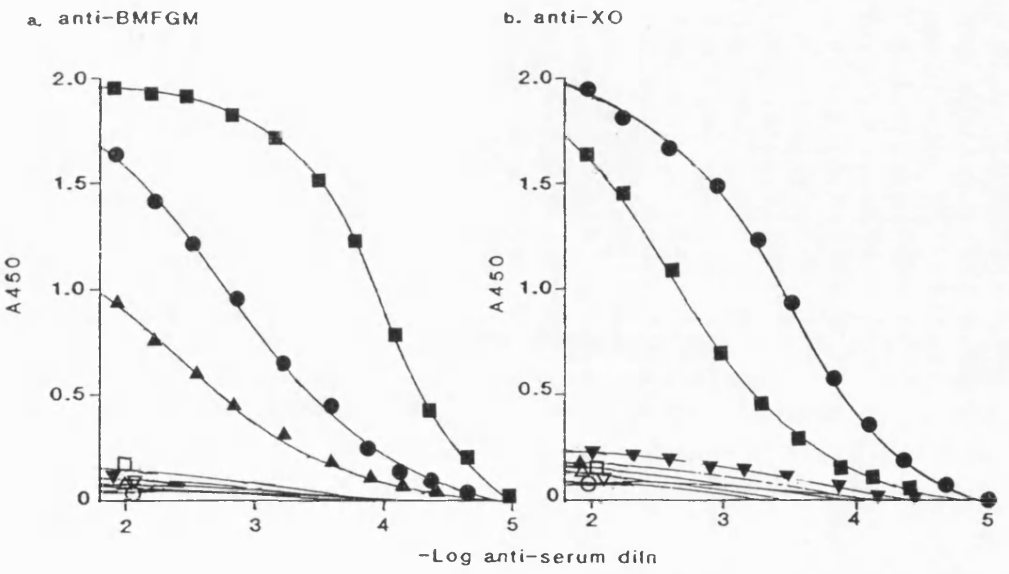
The unfilled symbols represent the pre-immune equivalents of the above anti-sera.

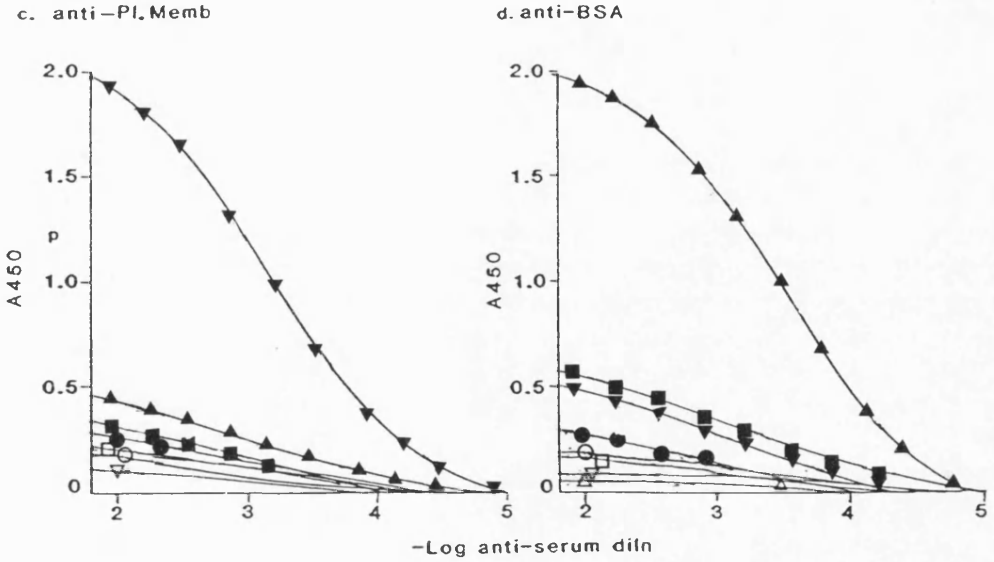
a. ELISA using BMFGM as coating antigen

b. ELISA using BXO as coating antigen

c. ELISA using PM as coating antigen

d. ELISA using BSA as coating antigen


a. anti-BMFGM
b. anti-XO
A450
2.0
1.5
1.0
0.5
0
2
3
4
5
-Log anti-serum diln


c. anti-Pl.Memb
d. anti-BSA
A450
2.0
1.5
1.0
0.5
0
2
3
4
5
-Log anti-serum diln

RABSA a weak response,and RABMFGM and RAXO no response at all.

iv An ELISA to detect anti-BSA antibodies was developed from the BMFGM ELISA (Materials and Methods Sect 3.3 (i), p86), by using BSA to coat the microtitre plates, in place of BMFGM (10µg protein/ml carbonate buffer). Again the same sera were assayed as in i, ii, and iii.

Fig 68d, p206, shows that RABSA gave a strong response, whereas RABMFGM, RAXO, and RAPM did not.

Pre-immune rabbit sera for all RABMFGM, RAXO, RAPM, and RABSA gave little response in any of the ELISA'S outlined above.

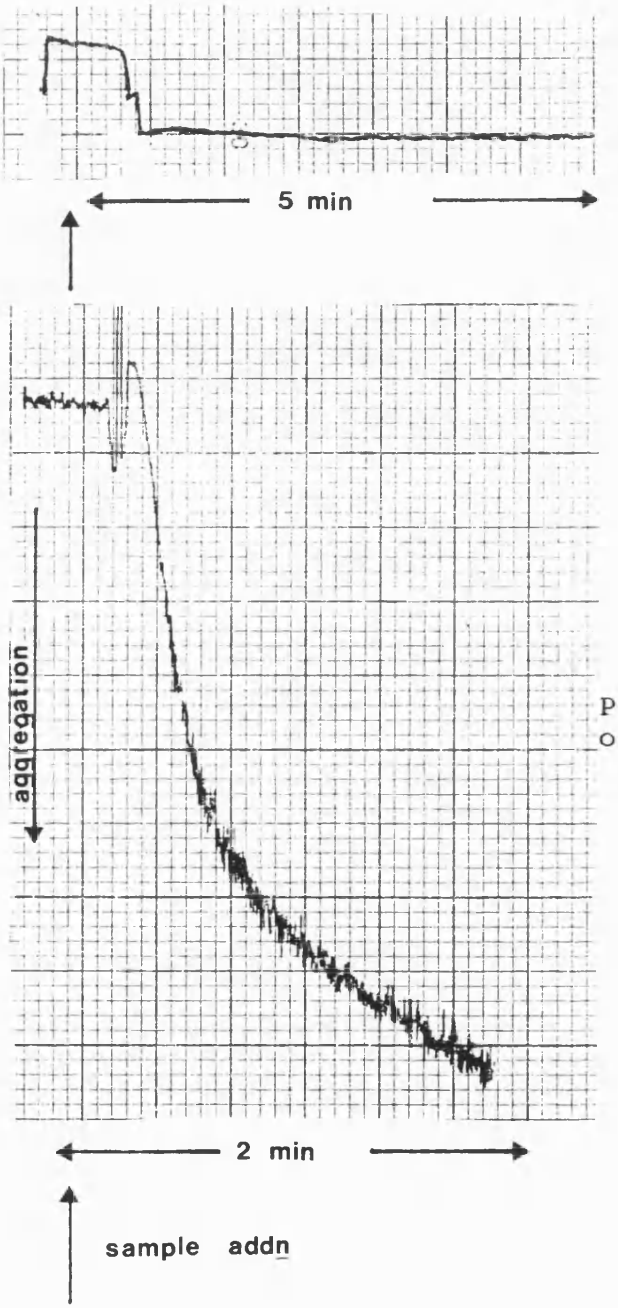
6.3 Platelet aggregation experiments.

Various pre-immune and post-immune rabbit antisera were available i.e. those prepared for the purposes of Sect 6.2.3 above and two others; Rabbit anti-Citrate Synthase (RACS), and Rabbit anti-Isocitrate Dehydrogenase (RAID), (provided by colleagues in the Biochemistry Dept). These antisera, along with their corresponding pre-immune sera were tested for their ability to aggregate freshly prepared, pooled PRP (Materials and Methods Sect 4.4, p96). Equal volumes of each (50 µl) were added to PRP (0.5ml).

Fig 69, p208, shows that all anti-sera tested caused platelet aggregation, whereas, no pre-immune sera had any effect.

Fractions of IgG were prepared from RABMFGM, RAXO, and

Fig 69 Platelet aggregation using a variety of pre-, and post-immune rabbit sera.

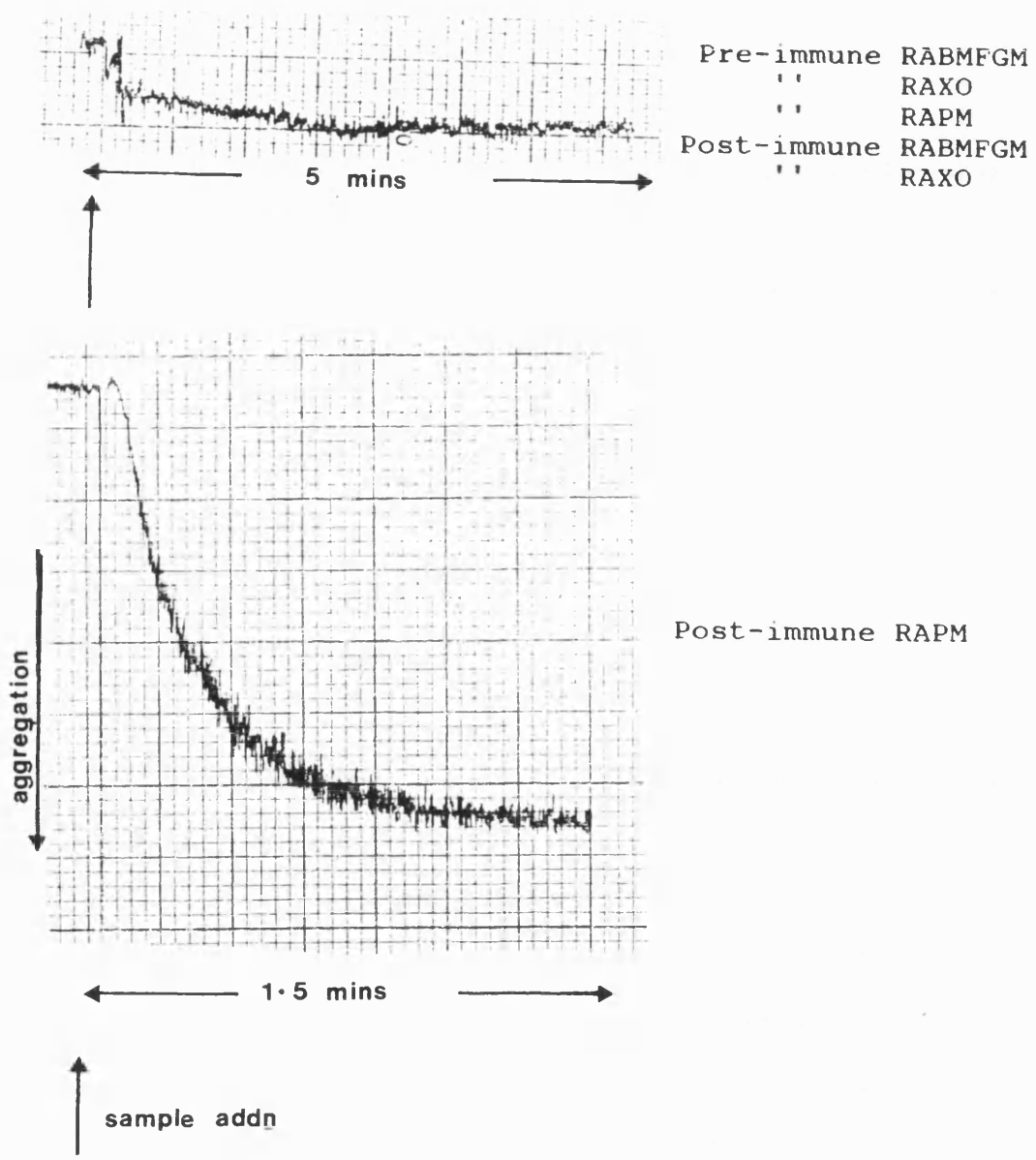


Pre-immune; BMFGM
XO
Pl. Memb.
BSA
Isocitrate DeH
Citrate Synth.

rabbit antisera.

Post-immune rabbit antisera of the equivalents above.

Fig 70 Platelet aggregation using pre-, and post-immune rabbit IgG from XO,BMFGM, and Platelet membrane (RABMFGM,RAXO,and RAPM).



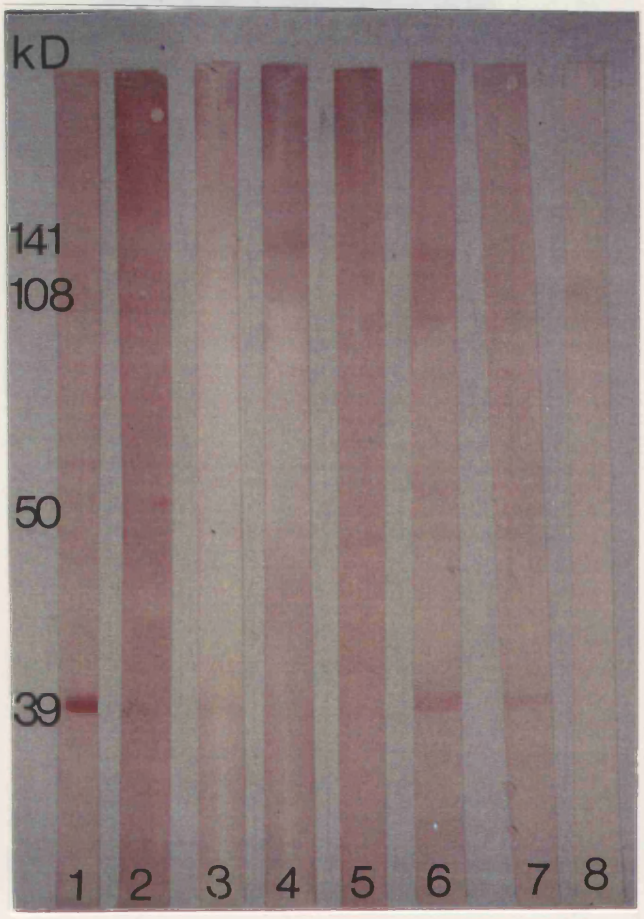
RAPM, as well as from their corresponding pre-immune sera (Materials and Methods Sect 2.5, p77). Each IgG preparation (50µl, 4mg/ml PBS) was added to washed platelets (0.5 ml), and platelet aggregation was monitored. Fig 70, p209, shows that only IgG prepared from RAPM sera caused aggregation whereas IgG prepared from RABMFGM, RAXO, and the three pre-immune sera had no effect.

6.4 Western blotting of SDS-PAGE patterns of Platelet membrane, followed by Immunoblotting.

Platelet membrane, prepared according to the method of Barber and Jamieson (1976), was subjected to SDS-PAGE (75ug protein / 100µl track) and transferred to nitrocellulose sheets. The transferred bands were incubated with either serum (1:25), or IgG (1:100), followed by detection using an appropriate conjugate (1:1000), Materials and Methods Sects 4.1 and 4.2, p89,93.

Human sera stained several bands when immunoblotted against SDS-PAGE patterns of human platelet membrane; prominent amongst these is a band at Mr 39,000 possibly corresponding to Fc receptor see Fig 71, p211. Affinity purified anti-BMFGM immunoglobulins showed little staining when immunoblotted against human platelet membrane see Fig 72, p212. Rabbit anti-BMFGM anti-sera stained a number of bands when immunoblotted against human platelet membrane, but many of these bands were also stained by other rabbit anti-sera, Fig 73, p213.

Fig71 Immunoblot of human sera against platelet membrane (using goat anti-human polyvalent HRP conjugate).



Titres of anti-BMFGM sera

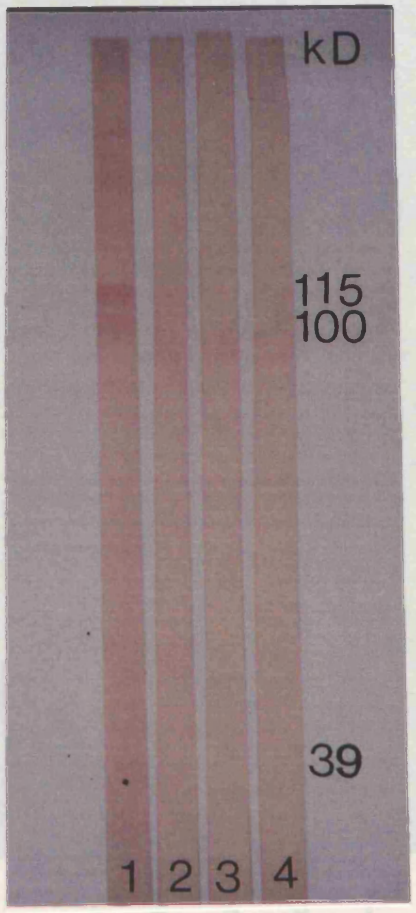
2,3. Low

5,6. Medium

1,4,7. High

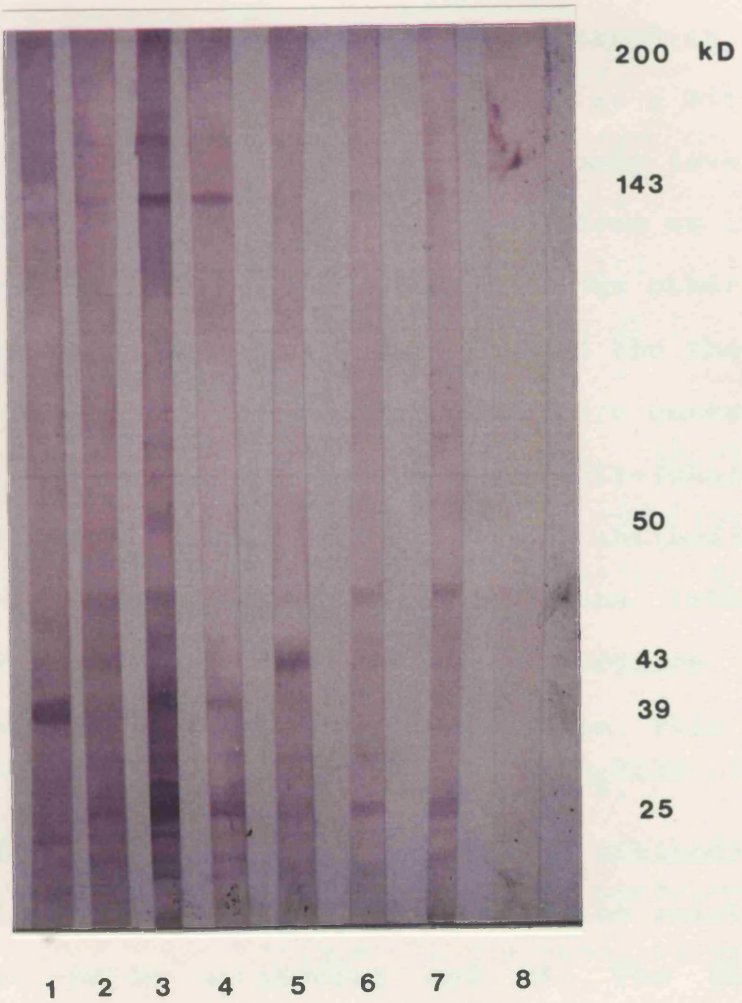
8. Blank (no sera)

Fig 72 Immunoblot profile of affinity purified anti-BMFGM antibody against platelet membrane (using goat anti-human IgG-HRP as conjugate).



1. Human IgG
2. Human IgG minus BMFGM antibody
3. BMFGM affinity antibody
4. Blank (no IgG)

Fig 73 Immunoblot-profile of rabbit anti-sera against platelet membrane (using goat anti-rabbit polyvalent antibody as conjugate).



1,2 Rabbit anti-BMFGM

3 Rabbit anti-platelet membrane

4- Rabbit anti-BSA

5- Rabbit anti-IDH

6- Normal Rabbit Serum

7- Affinity purified RABMFGM

8- Blank (no serum, or IgG)

DISCUSSION

DISCUSSION

Davies and coworkers (1974), showed a significant elevation of anti-(bovine whole milk) antibodies in the serum of MI patients, compared to controls; a difference that was even more striking when antibody levels were considered in patients who had died from an infarct. Their work was, however, refuted by various other groups (see Introduction Sect 1.5, p48) and the theory was largely discredited. After this literature controversy, Davies et al (1982), found that their anti-(whole milk) antibodies were essentially anti-BMFGM antibodies, and it now appeared possible that, if the latter were assayed directly, then differences between MI and control sera might be more clearly seen. This was the situation at the start of the present work.

An ELISA to detect anti-BMFGM antibodies was accordingly developed to investigate the relationship between anti-BMFGM antibodies and MI. The ELISA was optimised (Results Sect 1.1, p101) in terms of antigen (BMFGM) and conjugate concentrations. The assay was found to require minimal blocking; neither BSA, nor NGS were necessary. The effect of including casein was to reduce absorbances of all sera substantially. It seems unlikely that anti-BMFGM antibodies are absorbed out by casein, as they are known to be directed mainly against XO (see later), but it is possible that casein may bind to the membrane (Basch et al,1985), and block antigenic

sites, thus preventing the attachment of anti-BMFGM antibodies. In fact, some antibodies in human serum do bind to a protein on the BMFGM of the same Mr as casein (28kD), (see Results Sect 5.2, p160).

Serum dilutions were also examined (Results Sect 1.1, p102), to determine which gave the most consistent results, and assays were done on separate occasions to investigate reliability. Although absolute titres did vary slightly from day to day (Results Sect 1.3, p111), they were always within the same ranges.

Antibody binding to the BMFGM was also shown to be specific in that it was Fab' mediated and titres were independent of total IgG levels (Results Sect 1.2, p107).

Distributions of IgG anti-BMFGM antibodies in MI and Control sera.

By use of the BMFGM ELISA, initial comparisons were made of anti-BMFGM antibody levels (Results Sect 2.1, p114) in 462 "New" MI samples (<65 years of age, from South Wales and the Bristol area) and 517 healthy Control sera (49-64 years of age, from Caerphilly). No statistical difference was found between them. Anti-BMFGM antibody titre distributions in 602 "New", 401 6-month, and 428 2-year post infarct serum samples (ages 45-80, and from all regions of South Wales) were similar (Results Sect 2.2, p114). In fact, the only statistical difference observed was in low titres of anti-BMFGM antibody levels (i.e. < 10% Std, Results Sect 2.3,

p117), when antibody titres in 6-month and 2-year MI samples were actually found to be lower than those of the controls. This difference was not observed in the case of "new" MI samples. From this observation one might expect that new patient samples would have statistically elevated levels of IgG anti-BMFGM antibody levels (considering samples of <10% Std) compared to 6-month and 2-year samples. However, this was not the case (statistics not shown). Investigations on other batches of serum, carried out in the laboratory also showed that IgG anti-BMFGM antibodies are higher in control compared to MI samples, although this difference was never found to be significant (Benboubetra and Harrison, personal communication).

These observations were in conflict with Davies' hypothesis (1974) in two respects:-

1. IgG anti-BMFGM antibodies were not raised in MI patients compared to controls.

2. New patients did not have higher titres than 6-month or two year post-infarct patients, as would be expected if patients with high titres had a threefold increased chance of dying within the first six months after an infarct (Davies, 1984).

Origins of samples with respect to region, and age were examined, to see if any obvious differences within these parameters could explain this inability to repeat Davies' findings. However, IgG anti-BMFGM antibody levels were found to be similar in each of the five

regions investigated, and in all the age groups compared (Results Sect 2.5, 2.6, p123).

Distributions of IgG anti-BMFGM levels in some other disease states

Anti-BMFGM antibody levels were compared in other "autoimmune" disease states with those of healthy controls (i.e. males aged 49-64, as used in the MI studies). The anti-BMFGM levels in MND sera were very similar to those of the controls, whereas those of ITP and MG were elevated, but significantly only in the case of MG. A possible explanation for these findings may lie in the sex and ages of the samples. The incidence of MG is higher in women than in men, the ratio being 3:2 (Newsom-Davies and Vincent, 1982), and 80% of the samples tested were those of women. While details of the ITP donors were not available, patients, are mainly middle aged women, and chidren (George et al, 1985). MND sufferers, on the other hand, are mainly men with the overall proportion 3:2, but in some states of the disease can be 5:1 (Clifford-Rose, 1977). 90% of the MND samples tested were those of men. Rees (1985) reported that anti-BMFGM antibody levels are higher in women than in men, titres being elevated by pregnancy. Elevations of IgG anti-BMFGM antibodies have also been observed in the serum samples of children (Benboubetra and Harrison, personal communication). It is impossible to comment further about anti-BMFGM titres in MG or ITP

without data from age and sex-matched controls. In our studies with CHD patients, women have generally not been studied, in view of the likelihood (Rees, 1985) of the influence of hormonal changes. Concerning MG, it is worthwile noting that elevated levels of antibodies to other autoantigens commonly occur (Newsom-Davies and Vincent, 1982).

There were not enough samples to justify comment upon the relevance of anti-BMFGM antibodies in migraine sufferers; there appeared to be a distribution of low and high titres. In the light of evidence suggesting that migraine is linked with allergy (Monro et al,1984), it may be more prudent to consider anti-BMFGM IgE levels or those IgG subclasses particularly associated with allergy.

Comparison of ELISAs carried out at Bath and Carmarthen

Despite the fact that, in the above studies, Davies' hypothesis could not be verified, Rees and Thomas (West Wales Hospital, Carmarthen) were, in parallel studies, finding increases of antibody titres in MI samples compared to controls (in one of their studies 65% of MI and 35% of controls were positive for anti-BMFGM antibody, personal communication). This was particularly disturbing in view of the fact that their samples were from the same source as ours (Materials Sect 1.2, p69). It was decided therefore, to compare the two assays by systematically comparing antigens (Dried Milk v BMFGM), and conjugates.

On analysis, it became apparent that Carmarthens' use of a different conjugate (detecting IgG, IgA and IgM human antibodies, rather than IgG alone), was the major cause of the discrepancies between the two assay systems. Differences were found not to arise from different antigen preparations or from different treatment of samples (Results Sects. 3.1-3.3, p129-135).

The BMFGM ELISA was therefore modified to detect separately, IgG, IgA, and IgM and polyvalent (all three) anti-BMFGM antibodies by using commercially produced conjugates, in place of the laboratory-prepared anti IgG (Results Sect. 4.0, p135). IgG, IgA, and IgM anti-BMFGM antibodies were detected, and subsequently assayed in MI and control sera.

Preliminary analysis, on two sets of 65 "New" male MI and 65 male control samples (selected randomly and not matched for either age or region), showed, in each set, an elevation of polyvalent, and IgM anti-BMFGM antibody titres in MI patients compared to controls. No difference was found with IgG anti-BMFGM titres (as already observed in Results Sect. 2.1, p114), IgA titres in MI patients were decreased compared to controls. The difference in IgM anti-BMFGM titres was significant.

These results were verified when 100 MI and 100 control male samples were assayed as above. The MI samples this time were matched in age (45-64 yrs of age), and region (South Wales Valleys), with control samples (49-64 yrs of age, mainly from Caerphilly). In fact, anti-BMFGM titres (IgG) had already been shown not

to depend on either of these factors (Results Sect 2.5, 2.6, p123). Again, elevations of polyvalent and IgM antibodies were found in MI patients compared to controls. No change was observed in IgG and a slight reduction was observed in IgA antibody levels in the MI population. As before, IgM levels showed a statistically significant difference; the other differences were not significant. From the results of these investigations it was concluded that IgM anti-BMFGM levels were raised in "new" MI patients with respect to controls. These results agree with the Davies' findings, as outlined in detail below.

An Explanation of Davies' results

Davies' measured anti-BMFGM antibody levels by haemagglutination, which depends upon cross-linking of antigen-coated sheep red blood cells, by human antibodies (Boyden,1951;Rees,1973). All classes of antibody are detcted by this assay, but IgM, by virtue of its polyvalency, is particularly dominant (Roitt,1988). If anti-(BMFGM) IgM antibodies alone (i.e. not IgA, IgG etc.) are elevated in MI patients, then Davies' earlier finding can be explained, as can my own failure (Results Sect. 2.1 - 2.2, p114) to find differences between MI and control samples, by using a laboratory-prepared conjugate raised against human IgG (Results Sect. 3.3, p135). The later studies at Carmarthen, by Rees and Thomas, used anti-polyvalent conjugate, which detects IgG, IgA, and IgM antibodies.

These results can also be explained in terms of IgM anti-BMFGM elevation in serum. I, too, found an increase in anti-BMFGM antibodies in MI patients when using a polyvalent conjugate, although it was not statistically significant.

The above results clearly support the Davies hypothesis (Davies et al,1974), if this is restricted to IgM antibodies. Concerning the dismissal by other workers (discussed in Introduction Sect.1.5, p48), a number of points can now be made.

Oster et al (1974) verified Davies findings and used the same Haemagglutination assay, although Oster's choice of donors has justifiably been criticised (Carr et al,1975).

The results of Toivanen et al (1975a) are complicated by discrepancies in their Paper (see Introduction Sect 1.5, p51). Nevertheless, their figures from the text (i.e. average anti-milk titres from which negative titres have been excluded) do show an elevation of IgM antibodies in MI samples, as measured by radioimmunoassay. This is commented upon by Poston (1975). In fact, Davies (1984) had argued that the inability of Toivanen et al to repeat his findings arose from the latters' antigen preparation, which lacked the cream fraction of milk, containing the BMFGM.

Scott and coworkers (1976) also used the Haem-agglutination technique, but found no difference in antibody levels between MI and control groups. All samples were from male patients. Davies (1984) suggested

that the antigen (National Dried Milk) differed critically from that (Oster milk) used by his own group, but without detailed justification.

Gibney and co-workers (1980) used two methods to detect anti-milk antibodies; an ELISA using a conjugate raised against human IgG antibodies and also the Haemagglutination assay. Considering the fact that the conjugate used in the ELISA was against IgG, it is not surprising that no elevations of anti-milk antibodies were found in MI patients. In fact, as in my own studies, antibody levels appeared to be higher in the control groups. However, the fact that the Haemagglutination assay did not show any difference cannot be immediately explained. A large proportion of the controls were women (12/37) compared to the MI group (4/37). As already mentioned, Rees (1985) showed that anti-milk antibody levels in women are higher than in men and this factor may have served to elevate the antibody levels in the control group of Gibney et al. Alternatively, their antigen, "spray dried milk", may lack the antigens present in BMFGM.

Following the finding that IgM anti-BMFGM antibodies are elevated in MI sera compared with controls, it was clearly important to identify the BMFGM antigen more precisely.

The antigenicity of the BMFGM

To investigate further the antigenicity of the BMFGM it was decided to affinity-purify anti-BMFGM antibodies, particularly in order to characterise the BMFGM antigens by immunoblotting of its SDS-PAGE profile.

Attempts to purify anti-BMFGM antibodies from serum by using a batch absorption process were largely unsuccesful. Although it was possible to adsorb 100% of the anti-BMFGM antibodies onto the membrane, the maximum recovery was only 0.3 ± 0.1 %. Varying times of incubation and volumes of affinity eluting agent did not improve yields (Results Sect 5.1a, p148), although, changing the eluting agent from Gly/HCl to ammonium hydroxide boosted the recovery from 0.1% to 0.3% of original activity. Poor recoveries could not be explained by denaturation, either of the anti-BMFGM antibody or of the BMFGM itself (Results Sect 5.1a, p151).

In view of these failures, the use of an affinity column was investigated. The literature contains few methods for affinity purification of antibodies to membrane antigens. Steiner (1985) used a column containing immobilized platelets or platelet membranes to separate IgG directed to platelet surface antigens. His method of immobilisation of the membranes had been developed from that of Sela and Edelman (1977) who used glutaraldehyde fixed cells, immmobilised on Sephadex beads, to isolate immunoglobulins specific for cell surface glycoproteins. Following these procedures,

Sephadex beads were incubated with Concanavalin A followed by BMFGM and the cross-linking agent glutaraldehyde. This column matrix was then improved upon by using a commercially-available matrix to which Con A was already bound (Con A-Sepharose 4B). As the Con A content of this matrix (9mg/ml) was higher than that used initially (max. 1mg/ml), the column could be reduced in size from 50ml to 5ml. Nevertheless the % recovery of antibody improved and the yield was increased further by changing the cross-linking agent from glutaraldehyde to dimethylsuberimidate. Glutaraldehyde is a dialdehyde and theoretically could crosslink two proteins, via the ϵ-amino groups of lysine, by formation of Schiff's bases. The stability of proteins cross-linked by this method does not, however, agree with the known reversibility of Schiff's base formation, and an alternative mechanism has been proposed involving polymerisation products of glutaraldehyde (Richards and Knowles,1968). Glutaraldehyde has also been reported to react with N-terminal amino groups of some peptides, and the sulfhydryl group of cysteine (Habeeb and Hiramoto,1968). Dimethlysuberimidate also crosslinks proteins by covalent attachment to lysine groups but is more specific in its reaction and so less likely to bind to antigenic sites on the membrane, restricting anti-BMFGM antibody access. The column recovery was also improved by recirculating serum overnight. Following the above modifications, an affinity purification procedure was

developed which routinely yielded 15-20% of the original antibody activity. It was not found possible to generate serum, or IgG, free of anti-BMFGM antibody, by using this column. All flow through fractions contained approximately 25% of the original activity. This was not a consequence of overloading of the column (Results Sect 5.1b, p 156) and it may be that it reflects the presence of a fraction of anti-BMFGM antibodies with low avidity for BMFGM. Certainly, in the experimental situation, injection of large amounts of antigen will give rise to low affinity antibodies (Roitt,1988).

Sela and Edelman (1977) affinity-purified antibodies from serum and from an IgG preparation with equal success. Although this was also the case with the BMFGM column (Results Sect 5.1b, p156), after 5 purifications of serum, the column became blocked by a white precipitate. This was never experienced when IgG was used as a purification source. It appears that this precipitation is caused by some component of serum, possibly CICs or even IgM; IgM, in fact, was never detected in the affinity-purified fraction, although IgM anti-BMFGM antibodies clearly exist (Results Sect 4.0, p135).

A number of affinity-purified anti-BMFGM antibody batches were prepared; low titre sera produced low amounts of affinity purified IgG, as expected (Results Sect 5.1b, p160).

Information concerning the antigenicity of the BMFGM came primarily from Western blotting analysis of SDS-PAGE profiles of the BMFGM (Results Sect 5.2, p160). Human sera and affinity-purified antibodies bound to a band of approximately 150kD; corresponding to the enzyme, xanthine oxidase (XO). Higher titre sera stained more intensely than those of lower titre. Some lower MWt bands at 28, 36, 45, 59 and 66kD, were also stained, most noticeably in pooled sera. A polypeptide of 170kD was stained with some sera and with affinity-purified antibodies. It is possible that some of these stained bands result from milk serum proteins, adsorbed to the BMFGM. Casein (M.Wt 28kD), for example, is a known contaminant of the BMFGM (Basch et al, 1985), and anti-BSA antiserum was shown to cross-react with BMFGM (Results Sect 6.2.3, p204). The molecular weight of BSA is 67.5kD.

In contrast to human sera, which stained only a few bands in addition to that at 150kD, RABMFGM IgG immunostained most polypeptides of the BMFGM, as did the affinity-purified fraction.

These differences between rabbit anti-serum and human serum may indicate that:

1. Ingested BMFGM is the source of the human antibodies, but only a limited number of proteins, primarily XO, are absorbed and exposed to the immune system in immunogenic forms. SDS-PAGE profiles of dried milk which correlates with BMFGM as an antigen (Results Sect 3.1, p129) suggest that low molecular weight fragments are still

antigenic. The persistence of bovine milk XO activity has also been reported after gastric digestion in vivo and in vitro (Zikakis et al, 1976).

2. It is possible that the antibodies are, in fact, against endogenous XO, and fortuitously cross-react with the BMFGM.

Although IgG anti-BMFGM antibodies were affinity purified to increase the specificity of Western blots of the BMFGM, they did in fact give results comparable with those from whole human serum.

It will be interesting to see if IgM and IgA anti-BMFGM antibodies show the same response on immunoblot profiles of the BMFGM.

Investigation of anti-XO antibodies in human sera.

From the results, discussed above, it appeared that anti-BMFGM antibodies, at least the IgG fraction, were primarily directed against XO and so it was decided to assay anti-XO antibodies directly. For this purpose, an ELISA was used and, for this, purified XO was required. The enzyme prepared by the method of Nakamura and Yamazaki (1982) showed acceptable parameters and could be successfully used as antigen in ELISA for IgG and IgA anti-XO antibodies. Assay for IgM anti-XO antibodies was, however, complicated by high background responses in all sera tested. These background levels were found to be associated with the presence of a band on SDS-PAGE, of the purified XO, with M.Wt 83kD, which could be removed by further purification of XO on a folate affinity column (Results Sect 5.3, p167).

The receptor, secretory component, present on glandular epithelial cells is involved in mediation of transport of polymeric IgA and IgM into external secretions (Mostov et al, 1984). Two or three types of this receptor may be present in the animal. For the rabbit, polypeptides of MWt 77,80 and 83kD have been detected (Brandtzaeg,1974;Kuhn and Kraehenbuhl,1979). The bovine receptor is present on intestinal, liver, and mammary gland membranes. The membrane bound form has been reported to have a M.Wt of 93kD (Beale and Hopley,1986), and has been shown to bind bovine and human IgM. cDNA sequences predict a Mwt of 82,041D of this receptor (Mostov et al, 1984). If the 83kD contaminant in my XO preparation arises from this receptor, perhaps as a proteolytic fragment, it would explain why only IgM antibodies show a high background, in that total IgM binding to secretory component could be superimposed upon specific anti-XO binding. Although this receptor has not been demonstrated on BMFGM, it seems likely that, as secretory component is found on the mammary cell apical membrane, and BMFGM is derived from the latter, then secretory component will be present on the BMFGM also.

It might seem that the 83kD protein is most likely to be a proteolytic fragment of XO. Although Jarasch et al(1981) report a proteolytic fragment of XO, occuring at 85kD, other reports (Nagler and Vartanyan, 1976) have cited major proteolytic fragments on SDS-PAGE of 20, 42 and 92kD. In the present work, the 83kD polypeptide,

stained positively for carbohydrate (Results not shown), whereas XO did not and in view of the fact that cavine XO is known to contain no carbohydrate (Johnson et al,1985), it would seem that the 83kD band arises from another source.

ELISA comparisons of IgG, IgA, and IgM anti-XO and anti-BMFGM antibodies in the same serum samples showed significant correlations between anti-XO and anti-BMFGM titres (Results Sect 5.5, p173), further supporting the idea that XO is a major antigenic component of the BMFGM. The correlation coefficient was less when comparing IgM than with IgG and IgA levels; again, this may reflect non-specific binding by a secretory component-like receptor, in the case of the anti-BMFGM ELISA.

IgG, IgA, and IgM anti-XO antibody levels were compared in 100 MI samples and 100 controls (age and region matched).

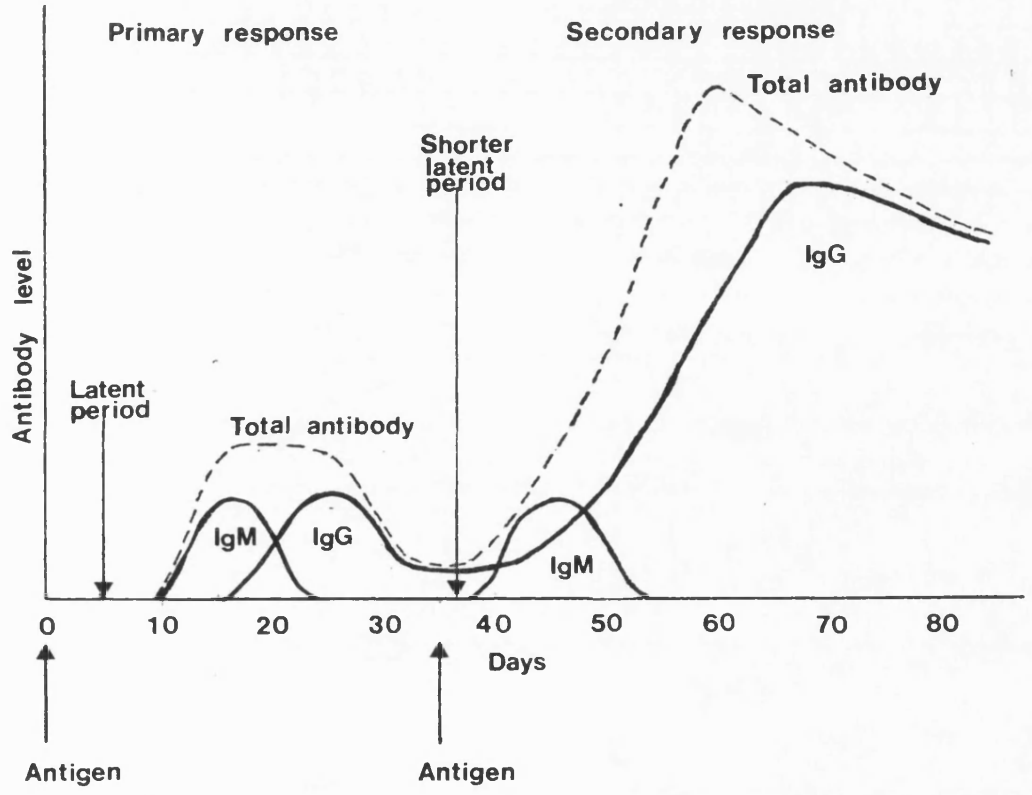
The results obtained were very similar to those obtained with 100 MI and 100 control sera tested for anti-BMFGM antibodies (Results Sect. 4.2, p144), again indicating that XO is the major antigen of the BMFGM. IgM anti-XO antibody levels were significantly raised in MI patients compared to controls, whereas those of IgG, and IgA were not (Results Sect 5.6,p173).

An alternative pool of MI sera was available from the Royal United Hospital (RUH), Bath, Avon. These samples had been collected, in general, within 24h, following an

MI, at the Coronary Care Unit; this was much earlier than the 15-30 days that were usual for the above South Wales samples. These RUH samples (56) were compared with controls (44) who had been hospitalised, with no evidence of heart or liver disease, or cancer. A highly significant increase in IgM antibodies was seen in MI patients; this was more significant (p = 0.1%) than the above analysis (p = 5%) of South Wales samples.

Concerning the RUH 24h post infarct samples, it is unlikely that an elevation of IgM anti-XO antibodies resulted from the infarct itself. Fig 74, p231, shows that, for a primary response to antigen, IgM antibodies can take up to 10 days to appear, and after a second exposure to antigen take about 3 days. It is possible, of course, that an IgM response could have occurred to a sub-clinical event, preceding the heart attack. Alternatively, it may be that IgM anti-XO antibody levels are permanently elevated in those people susceptible to MI. The MI samples from South Wales, as had been stated, were taken 15-30 days post-infarct and still showed elevation of IgM anti-BMFGM and anti-XO levels compared with controls (Results Sect 4.2 and 5.6, p144,173). At this stage IgM antibodies generated in response to the MI might well be expected to have fallen away with time and indeed IgG levels would be probably be elevated; this was never observed. Overall, the implications are that the immunological disturbance we are seeing does not result from initial exposure of an antigen.

Fig 74 The Primary and Secondary antibody responses.



From Kirkwood and Lewis (1989), p29.

It is true that the elevation of IgM levels in MI patients was more significant in more recently infarcted patients and this could reflect a slow decrease in anti-XO IgM levels after infarct or pre-infarct events. Alternatively this higher significance may simply be the result of more stringent selection of control subjects in the RUH samples. The controls available from the Caerphilly-Speedwell study were taken from the population at large and, inevitably, some of these samples would be from donors with some previous evidence of Ischaemic Heart Disease. This could not conveniently be checked. Time did not allow assay of either IgA, or IgM anti-BMFGM antibodies in 6-month or 2-year samples, to see whether or not the elevation of IgM antibodies could be detcted in these samples, with respect to controls. Certainly, IgG anti-BMFGM levels were shown not to change when comparing "New" with 6-Month samples and 6-month with 2-year samples.

General antibody levels and Myocardial Infarction.

It has been shown that a biphasic response exists in the IgG fraction in some patients (30/45) after an MI (Ebringer et al,1971). An initial fall, with lowest level at day 5, is followed by an increase. By the fourth week after an MI, serum IgG levels return to normal (Finkelstein et al,1963). Serum IgA, and IgM have been reported not to change (Ebringer et al,1971). In Myocardial Ischaemia, however, there is no obvious fall or rise in IgG levels, but the average IgG level is

significantly higher than in controls (15.48 mg/ml compared to 12.59 mg/ml). For MI patients, this average is 12.65 mg/ml. Gray et al (1978) have reported an increase in levels of total IgG and IgM in patients with old proven infarcts, while the level of IgM was raised in patients with peripheral vascular disease. Interestingly, total IgE levels have been reported to be elevated in MI patients (Criqui et al, 1987) (in contrast to IgG), and to increase gradually after infarction, reaching a sustained maximum by Day 7. This is followed by a drop to initial levels by the end of week 3. In some patients, IgE elevation persists (Szczeklik et al, 1988).

Autoantibodies against cardiac tissue are known to appear in the 2nd and 3rd weeks after an MI (Ehrenfeld et al, 1961), and eventually to decrease in most patients (Heine et al, 1966). This coincides with an overall elevation in antibody levels as discussed above. Post-cardiac injury syndrome, frequently found after cardiac surgery (Engle and Ito, 1961), and in 0-6% of acute MI cases, is associated with actin and myosin autoantibodies (De Scheerder et al,1985). IgG antibodies to cardiolipin are also reported in young post MI patients, and are high risk markers for recurring events (Hamsten et al,1986). Interestingly, Davies and coworkers, 1974 report an attempt to measure antibodies against extracts of human heart, but only 5/53 were positive, and in these cases, titres fell with recovery, in contrast to the persistent elevation of anti-BMFGM

antibody levels subsequent to infarct (Davies et al, 1974).

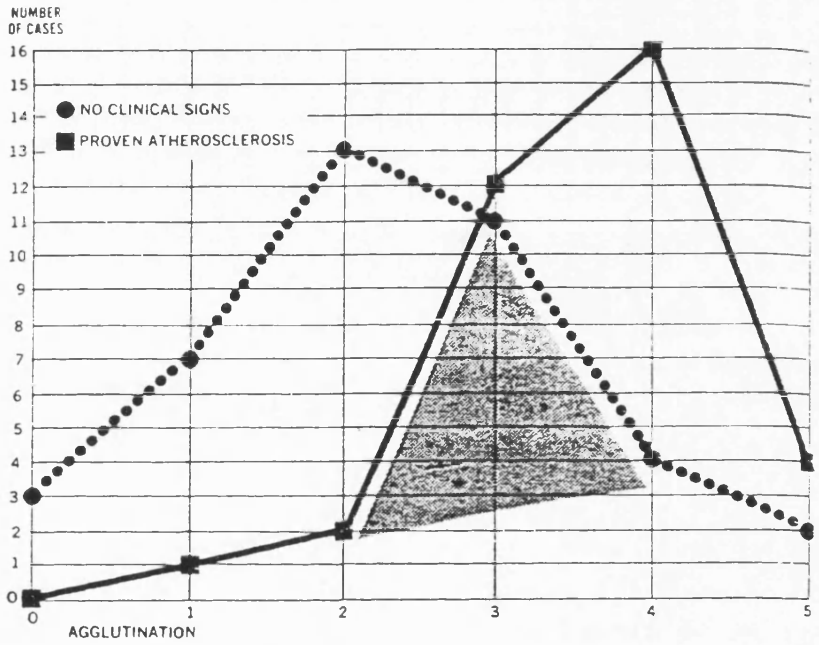
Work reported here (Results Sect 1.2, p107) showed that IgG anti-BMFGM antibody levels do not correlate with total IgG levels, and so are not necessarily affected by the patterns mentioned above.

Anti-XO antibodies in human sera

Anti-XO antibodies in human sera have been documented elsewhere and not in the context of heart disease. Jarasch et al, (1986) showed high levels in normal human serum, estimating that they can account account for 0.2-8% of the total IgG. However, only antibodies of the IgG class were reported and no association with any disease state was found. In the present work, levels of affinity-purified IgG-BMFGM antibodies, ranged from 0.08mg/ml to 1.17mg/ml (Results Sect 5.1b,p159). If one assumes an average total IgG count of 11-12mg/ml (Johnstone and Thorpe, 1982), and that most of the affinity purified antibodies are directed against XO, then this agrees very well with the data of Jarasch et al. Experimental antibodies to bovine XO have been used to show that most of the XO present in bovine organs is present in the BMFGM and endothelial cells of small capillaries and vessels (Jarasch et al, 1981).

Oster and coworkers too (1974), reported anti-XO antibodies in human sera and, in fact, found that levels (assayed by haemagglutination) were elevated in atherosclerotic patients (see Fig 75, p235).

Fig 75 Immunoassay of serum xanthine oxidase.



From Oster et al, 1974

This and related reports are discussed in more detail below.

Xanthine Oxidase and Heart Disease.

The main aim of the above paper was to support Oster's hypothesis that ingested bovine XO finds its way into the human bloodstream. Oster (1968) advanced a novel mechanism for human atherosclerosis. He postulated that XO, derived from bovine milk, may be deposited in arterial and myocardial walls, and, by oxidising fatty aldehydes, lead to depletion of membrane plasmalogens. The resulting membrane damage would stimulate cell proliferation, scar formation, cholesterol ester deposition and ultimate development of the atherosclerotic lesion (Oster, 1971,1972,1973a).

Earlier work (Oster and Mulinos, 1944) had shown that organs normally devoid of plasmalogens i.e. the liver and small intestine, had an abundance of XO. The normal human myocardium contains little or no XO (Eddy et al,1987) but XO was, nevertheless, reported in atherosclerotic lesions, and in the infarcted myocardium (Ross et al, 1973). Moreover Oster and Hope-Ross (1966) found that, in the case of a fatal MI, plasmalogens disappear from the infarcted area, 2h after the onset of pain. They surmised that the pain of an MI was produced by plasmalogen depletion in the myelin sheath of nerve terminals in the heart (Oster and Ross, 1975). Further support for Oster's theory came from observations that patients with clinically manifest atherosclerosis

obtained relief by administration of the XO inhibitors allopurinol (Oster, 1968), and folic acid (Oster 1973b).

Oster concluded that the most likely source of XO was dietary. Cows' milk and certain dairy products contain the active enzyme in abundance (Zikakis and Wooters, 1980), and activity may be retained during digestion (Zikakis et al,1976). Oster argued that human anti-XO antibodies may represent attempts by the body to inhibit the enzyme (Oster,1976). Oster (1976) proposed that milk fat globules containing XO can readily cross the intestinal barrier, and that the XO may be active. Homogenisation of milk creates microglobules, and after pasteurisation, as much as 66% of XO may still be in its active state (Greenbank and Pallansch,1962), [18% according to Zikakis and Wooters (1980)]. Oster concluded that "the dietary origin of the atherosclerotic lesion is proportional to the biological availability of XO in youth".

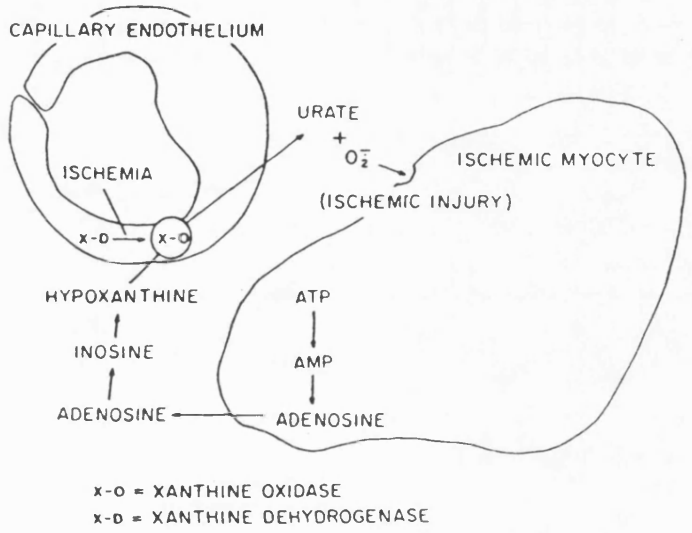
Reports by the F.D.A.(Carr et al,1975) and Deeth (1983) concluded that the evidence supporting Oster's hypothesis was inconclusive. Many aspects of his work were criticised, particularly his evidence that active bovine XO could enter and survive in the bloodstream (Volp and Lage,1977). Although retention of enzymic activity may remain in doubt, it has become clear that ingested large protein molecules do reach the bloodstream (Introduction Sect. 3.0, p60) and it has been shown that titres of anti-XO antibodies increase

with increasing milk consumption (Rzucidlo and Zikakis, 1979), however serum XO activity itself does not appear to increase with increasing milk consumption (McCarthy and Long,1976).

In recent years, interest in the possible involvement of XO in heart disease has arisen for apparently totally different reasons. Endogenous XO has been proposed to be a source of oxygen radicals (Hearse et al,1986) which damage myocardial tissue during a period of re-oxygenation after a hypoxic period (Granger et al,1981;Chambers etal,1985; McCord 1985). XO exists in mammals in two forms (Battelli,1980), a dehydrogenase form (D-form) which uses NAD^+ as electron acceptor, and an oxidase form, using O_2 as the acceptor. During a period of ischaemia, xanthine oxidase (D-form), is rapidly converted to the oxidase form (Chambers et al,1985;Engerson et al,1987) which will reduce molecular oxygen to either superoxide or hydrogen peroxide (Link and Riley,1988).

The breakdown of ATP in the ischaemic heart causes accumulation of hypoxanthine, a substrate for XO while reperfusion provides the O_2 (Eddy et al,1987; Abd-ElFattah et al,1988) see Fig 76, p239. Several recent studies indicate that allopurinol and oxypurinol, competitive inhibitors of XO are protective in the reperfused dog heart (Charlat et al,1986;Hearse et al,1986;Werns et al,1986), although other workers dispute these findings (Puett et al,1987;Kinsman et al.1988).

Fig 76 The proposed mechanism by which xanthine oxidase in the heart can serve as a source of oxy-radicals during ischaemia/reperfusion.



From Downey et al, (1988).

However, the relevance of this hypothesis to the human myocardium remains controversial. Several species, including rabbits and pig have undetectable amounts of myocardial XO. The XO content of human myocardium is unclear, reported amounts varying from trace amounts to excess (Eddy et al, 1987). As mentioned above, it has been reported that XO is confined to the capillary endothelial cells of heart muscle, at least from a bovine source (Bruder et al,1983; Jarasch et al,1981).

IMMUNOLOGICAL CROSS-REACTIVITY BETWEEN THE BMFGM AND PLATELET MEMBRANE

Davies and Rees anticipated that antibody to the BMFGM would cross-react with other cell membranes, including possibly the human platelet membrane, long suspected of playing a role in atherogenesis.

Davies and coworkers (1982) demonstrated evidence of such cross-reactivity as follows: They showed that an immune serum raised to BMFGM in rabbits caused aggregation of platelets, recorded both visually, and by means of a platelet aggregometer, whereas pre-immune serum had no effect. Also rabbit anti-BMFGM antibody, in post-immune serum was shown, by immunofluorescence, to bind to platelets, whereas that from pre-immune serum had no effect (Davies,1984;Rees,1985).

Rees (1985) hypothesised that anti-BMFGM antibodies may bind to platelets, thus assisting their removal from the circulation. This would effectively increase platelet turnover and there would be a greater

proportion of fresh, active platelets in the circulation. It would follow therefore that MI patients, with high levels of anti-BMFGM antibodies, would have higher levels of active platelets. This, in fact, was demonstrated by Rees (1982).

In the present studies, it was decided to investigate these proposed cross-reactivities in more detail.

The platelet membrane is now thought to contain over 120 different proteins; no great surprise considering the many functions it must fulfil (Capron et al,1985;George et al,1985). Although the platelet is not considered an effector cell of the immune system, it is known to react with antibodies in different ways. Platelet antigens can be divided into two groups:-

1. Platelet-specific antigens - these make platelets susceptible to attack by antibodies arising spontaneously, e.g. post-transfusion and in pregnancy (Shulman,1964;Von dem Borne,1980).
2. Non-platelet specific antigens - e.g. the ABO system (Moreau and Andre, 1954), HLA (Aster et al,1973), Tn and T (Cartron and Nurden,1979), Senescent (Kay, 1981), and Fc receptors (discussed below)

Human platelets are known to possess an Fc receptor for IgG, FcγR (Israels et al,1973; Karas et al,1982), and for IgE (Capron,1985).

Three different types of FcγR have been distinguished by using monoclonal antibodies (Anderson and Looney,1986):-

FcγRI - (72kD), found on monocytes,
FcγRII - (40kD), found on neutrophils, monocytes, eosinophils, platelets and B-cells,
FcγRIII - (50-70kD), found on neutrophils, large granular lymphocytes and macrophages.

Because of the large volume of platelets in the blood, these constitute the major source of the Fcγ RII receptor. The blood concentration of this receptor, provided by platelets, is 0.7nM (0.22nM by neutrophils, 0.014nM by monocytes and 0.02nM by B-cells). The receptor has a low affinity for monomeric IgG and preferentially binds immune complexes (Karas et al,1982). Immune complex-induced aggregation in platelets appears to reach maximal intensity when the immune complex is formed from a four fold antigen excess and up to 20 fold antibody excess (Battersby et al,1984). All sub-classes of human IgG activate human platelets whereas IgM, IgA, and IgD or bovine IgG do not (Pfueller and Luscher, 1972; Henson and Spielberg, 1973).

The role of FcγRII is thought to be to aid removal of CICs from the circulation by delivery to the reticulo-endothelial system.

It is possible, therefore, that anti-BMFGM antibodies may bind to platelets by means of their Fc portion, and not via their Fab' portions as originally anticipated by Davies and others (1982).

Between 8,000 and 50,000 molecules of IgG per platelet have been detected on platelet membranes and in platelet lysates (Kelton and Gibbons, 1982). It has been assumed that they were mainly Fc associated. However, it has been shown that IgG is also contained in platelet α-granules (George and Saucerman,1988).

Development of a platelet ELISA

An ELISA was developed to meaure anti-platelet membrane antibodies. The method of Horai and co-workers (1981), involving the use of monolayers of whole platelets as antigens was found to be unreliable for two reasons: the first was that a high background could not be eliminated (i.e. conjugate was binding non-specifically); the second was that results were variable and no standard curve could be produced. Thomas (West Wales hospital, Carmarthen) reported similar findings (personal communication). It was thought that loosening of the platelets adhering to microtitre wells might occur during the washing procedures and lead to inconsistencies, but the use of poly-l-lysine, or Ca^{2+} in the ELISA buffer did not improve matters. Platelets are known to contain surface IgG and it was considered possible that these were contributing to the high

background levels, but pre-washing of the platelets did not lead to improvement (Results Sect 6.1a, p185).

In view of the lack of promise of the above assay, the ELISA of Winiarski and Ekelund (1986), which uses preseparated platelet membranes, was further investigated and developed as follows:-

1. The results of Winiarski and Ekelund had suggested non-specific binding of sera, as titres produced by using serum or $F(ab')_2$ were not always comparable. NRS was accordingly included in the buffers and this was found to reduce the effect (Results Sect 6.1(iii), p187).

2. The membrane preparation, used by the above authors, involved sonication which, although very quick, did not produce consistent results. It was shown (Results Sect. 6.1b, p191), that the SDS-PAGE profile of the platelet membrane varied with sonication time and, more subtly, preparations showing identical SDS-PAGE patterns, gave different background levels in the ELISA. Sonication has been reported to cause membrane damage (Freifelder, 1982). The method of preparation of platelet membranes, described by Barber and Jamieson, (1970), was found to be an improvement and produced a consistently low background when used to coat ELISA microtitre wells.

3. The original ELISA used 50µg membrane protein/ml coating buffer; this was reduced to 10µg/ml. It is possible that the authors' method of measuring membrane protein was not as sensitive as that used in the laboratory, which was specifically modifed for membrane

protein estimation (Methods Sect 3.1(ii), p84).

4. Carbonate buffer, used in coating the platelet membrane onto the microtitre plates, was found to give a wider range of absorbances, compared to using formaldehyde to coat platelet membranes to wells. It may be that formaldehyde interferes with antibody binding to membrane.

The platelet ELISA was used in various ways to show that affinity purified anti-BMFGM antibodies did not bind specifically to the platelet membrane and that anti-BMFGM antibody titres did not correlate with anti-(platelet membrane) antibody titres (Results Sect.6.2.1, p197). In attempts to demonstrate the validity of the ELISA for anti-(platelet membrane) antibodies, ITP sera were compared with controls. Although ITP sera did show higher responses than controls, the difference was shown not to be significant (Results Sect 6.2.1, p197). There is some controversy, however, over the role of anti-platelet antibodies in ITP (van Leeuwen et al,1981), as some workers support a role for immune complex involvement (Trent et al,1980;Walsh et al,1984).

If time had allowed it would have been more appropriate to prepare IgG fractions from sera to assay, as then there would have been no interference from immune complex binding, and specific anti-(platelet membrane) binding would have been observed.

Demonstration of the lack of specific immunological cross-reactivity between BMFGM and Platelet membrane

Incubation of platelets with human serum could be shown to inhibit the BMFGM ELISA, but this inhibition was largely removed when the platelets were pre-blocked with normal rabbit serum, suggesting that the inhibition was largely the result of immune complex binding (Results Sect 6.2.2, p201). Platelets used as inhibitors in the platelet ELISA showed only a slight loss of inhibition when pre-blocked.

Experimental antisera also showed no cross-reactivity between anti-BMFGM and human platelet membrane (Results Sect 6.2.3, p204). Rabbit anti-BMFGM and anti-XO antisera showed no response in the platelet membrane ELISA and conversely, anti-(platelet membrane) antisera showed no response on either the BMFGM or the XO ELISAs. Rabbit anti-BSA antisera did respond slightly in the BMFGM and platelet membrane ELISAs suggesting that BSA may be on the BMFGM and a cross-reacting protein, possibly human serum albumin, may be present on the platelet membrane.

Immunoblotting of human serum onto SDS-PAGE profiles of platelet membrane highlighted a band at 39kD, likely to be the Fc receptor. Affinity-purified anti-BMFGM IgG gave little staining, and RABMFGM sera only stained bands that were also stained by other experimental antisera. No evidence of any antigenic determinants, as seen with immunoblot profiles against the BMFGM, was evident.

Certainly, no cross-reacting protein of 150kD was present on the platelet membrane.

One of the experiments cited by Rees (Davies,1984; Rees,1985) as evidence of antigenic cross-reaction of the BMFGM with the platelet membrane was repeated. He found that the addition of post-immune rabbit anti-BMFGM antisera aggregated platelets, whereas serum from pre-immune rabbits did not. In the present work, a variety of other pre- and post-immune sera were tested in the same way (Results Sect 6.3, p207), and it was shown that platelet aggregation occurred for all post-immune sera tested. It seemed unlikely that the platelet membrane cross-reacted with all of these antigens. This platelet-induced reaction most probably resulted from immune complex formation, as all of the anti-sera were collected 5-6 days after injection of the animal with antigen (see Fig 77, p249, in fact the lag phase of appearance of antibody would be even shorter, than shown here). In support of this idea was the finding that IgG purified from anti-BMFGM and anti-XO antisera did not aggregate platelets, whereas IgG prepared from anti-platelet membrane antisera did.

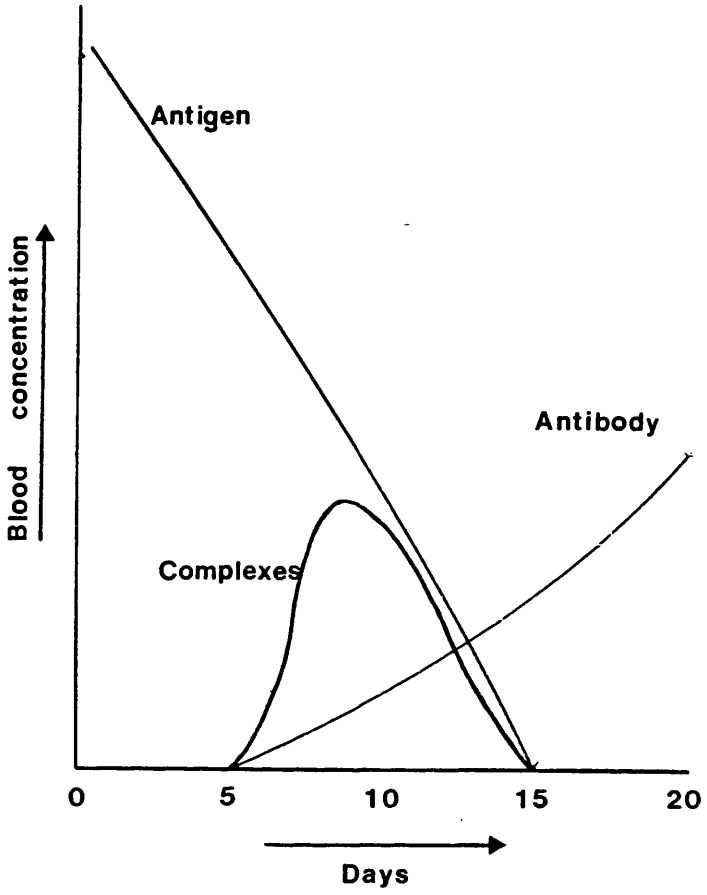
A pilot experiment, not described in the Results section showed that the addition of whole dried milk, to equal volumes of PRP, from different samples, resulted in aggregation of some PRP and not others. Subsequently, the plasma was separated from platelets for each sample and assayed for IgG anti-BMFGM levels. Interestingly, out of the 25 samples tested, the 6 samples that

contained platelets which aggregated had the highest anti-BMFGM titres. This would be consistent with formation of immune complexes, in the high titre plasma, that bring about aggregation.

It is possible that, depending on relative concentrations of anti-BMFGM (XO) antibody and antigen, CICs may be formed in vivo. Certainly anti-BMFGM (XO) antibodies constitute a large proprtion of total IgG. CICs have been reported to be increased in MI patients (Fust et al,1978;Smith et al,1983); an elevation that apparently does not result from neo-antigen formation from the myocardium (Smith et al,1983). These complexes contain IgG, IgA, and IgM antibodies (Triolo et al,1984). Increased levels of CICs, by binding to platelets, are likely to accelerate turnover of the latter (Rees, 1985) and to raise the levels of fresh, more active platelets. An increase of platelet activity in MI patients is well documented (Tofler,1987;Elwood,1988). This could explain increases in platelet adhesiveness (Davies and Lloyd 1967).

Davies (1958) also reported reductions in the RBC migration times of MI patients. Although RBCs do not have an Fc receptor to bind CICs, they have a high affinity receptor, CR1, for a complement component C3b, often bound to soluble CICs. CR1 binds C3b-CICs and serves in the transport of the complexes in the circulation to the sites of their elimination in the liver (Medof,1982;Cornacoff et al,1983). CIC bound RBCs would have reduced migration times.

Fig 77 Formation of soluble immune-complexes after injection of a large amount of antigen, as antibody is first synthesised.



From Roitt (1977), p171.

CONCLUDING REMARKS

CONCLUDING REMARKS

This final section attempts to summarise the progress made in the course of this work, to outline the many remaining uncertainties and to suggest how these might be resolved.

At the start of the project, Davies' hypothesis, linking anti-(cows' milk) antibodies with MI was largely discredited. There were however, reasons to question the validity of refuting publications and to believe that a repeat of Davies' experiments, with certain modifications might lead to more clear cut differences between MIs and controls. Although, in several surveys, Davies had consistently found elevated antibody titres in MI patients, these elevations were not always shown to be significant. He, himself, felt that significant elevations were most likely to be found when patients sera were assayed soon after the MI, i.e. before those with higher titres died. Another factor was Davies' later finding that anti-(cows' milk antibodies), as assayed by him, were largely anti-BMFGM antibodies. In view of these considerations, it seemed likely that a careful analysis of anti-BMFGM antibody titres, assayed by ELISA, rather than the less precise haemagglutination method, in patients with recent MIs might show more clearly any true differences from controls.

Accordingly, a large scale study was carried out, in conjunction with the MRC Epidemiology Unit at Cardiff, assaying IgG anti-BMFGM antibodies, but, surprisingly at the time, MI titres were not shown to be higher than those of controls. However, subsequent smaller numbers of subjects, showed that IgM anti-BMFGM antibodies were significantly elevated in MI patients. This finding, to a large extent, confirms Davies' results and explains differences between his data and those of dissenting groups.

The question then arose as to the nature of the immunogen giving rise to human anti-BMFGM antibodies. Western blots of human sera and affinity-purified anti-BMFGM antibodies on SDS-PAGE patterns of BMFGM pinpointed the enzyme, xanthine oxidase, as the major antigenic target. In accordance with this, two separate, carefully controlled studies showed IgM anti-XO antibodies to be significantly elevated in MI patients.

Having established a clinical significance of raised anti-BMFGM antibody levels and the association, even identity, of these antibodies with anti-XO antibodies, it remained to examine Davies' and Rees' belief that anti-BMFGM antibodies might interact with human platelets with pathogenic consequences. The overall conclusions, from competition ELISAs, Western blotting and platelet aggregation experiments was that there was no specific antigenic cross-reactivity of this type, although it may be that anti-BMFGM antibody-containing immune complexes bind non-specifically to platelets and effectively activate them.

These results serve to explain most of Davies' findings and those of relevant papers from other groups. The basic question, of course, concerns the origin and role of human anti-XO antibodies. The presence, in most humans, of circulating IgG anti-XO antibodies has been reported (Oster et al,1974;Bruder et al,1984;Jarasch et al,1986). The work in this thesis confirms their presence in remarkably high levels and additionally established the occurance of IgA and IgM anti-XO antibodies. Most importantly, the latter antibodies were found to be elevated in patients following MI*.

Anti-XO antibody levels have been reported to correlate with ingestion of milk products (Rzucidlo and Zikakis, 1979), and bovine milk would seem to be the obvious source of such antibodies. Oster's hypothesis of initiation of atherosclerosis had depended on the presence, in the circulation, of bovine milk-derived xanthine oxidase and it was the likliehood of this that was mainly attacked by the critical FDA report (Carr et al,1975). In fact, studies by others (Warsaw et al,1975) have established that proteins of up to 80kD commonly find their way through the gut, even of adults. It is quite conceivable that, at least, proteolytic fragments of bovine milk-derived XO could be absorbed from the gut and provoke an immune response. Oster (1976) argued in favour of the absorption of whole fat globules with BMFGM but my own data would suggest otherwise, in that Western blots of human antibodies showed few

*see Appendix

polypeptides in addition to XO itself. If the entire BMFGM had constituted an immunogen, then many more bands as shown with rabbit anti-BMFGM antisera, might be expected.

A number of groups have failed to demonstrate the presence of significant amounts XO activity in normal human serum (Shamma'a et al,1973;McCarthy and Long,1976). XO activity, however, is known to be elevated in acute liver injury (Giler et al,1975) but not in MI, as measured by these workers. It may be that only inactivated enzyme or proteolytic fragments find their way into the bloodstream. Alternatively, in view of the high levels of anti-XO antibodies, it is possible that any free XO is rapidly cleared in the form of circulating immune complexes.

It does appear conceivable that digested bovine XO could give rise to the anti-XO antibodies, clearly abundantly present in blood. It is also conceivable that active XO, in the form of immune complexes, could be deposited in blood vessels and initiate endothelial membrane damage; possibly not by plasmalogen depletion, as propsed by Oster (1971), but by generation of free radicals. Antibodies which bind to XO do not reduce all of the enzymes' activity (Ultmann et al,1962; Bruder et al,1984). The XO detected by Oster and coworkers (Ross et al,1973) in atherosclerotic plaques might have arisen in this way. It could be argued that those individuals who absorb higher levels of XO generate higher levels of anti-XO antibodies and have higher risk of pathogenic

effects from active immune complex deposition. Why then is correlation of anti-XO antibody levels and MI seen for IgM but not IgG ?

IgG anti-XO antibody titres are considerably higher than those of IgM antibodies. On the other hand, IgM antibodies are more efficient in immune complex formation (Roitt,1988). It may be that those individuals who, for some reason, generate and maintain higher IgM responses to bovine XO are particularly at risk.

The above discussion does not consider endogenous XO, which is certainly present in human liver and intestine (Parks and Granger,1986). It may be that the particularly high and prevalent levels of anti-XO antibodies, in most humans, arise through bovine XO-induced breaking of tolerance to autoantigens, as has been proposed for viral or bacterial induction of certain autoimmune diseases. Bruder et al (1984) suggest that two populations of anti-XO antibodies may exist in human sera: The first against bovine XO; the second against human XO.

Their evidence for endogenous origin of anti-XO antibodies is that various animal species produce anti-XO antibodies; and bovine IgG anti-XO antibodies do not cross-react with the human form of the enzyme. For evidence of a bovine source of the enzyme, they show that antibodies are also present in serum which cross-react with other proteins of the BMFGM that human IgG anti-XO antibodies will precipitate both human and bovine XO from the MFGM, and that some sera bind BXO with greater affinity than human XO from milk globules.

Despite the widespread occurrance of such antibodies and their quantity in sera, unlike any other auto-antibody (Guilbert et al,1982), they support the hypothesis that XO antibody formation is due to a self-immunisation of the enzyme, being a multiple event. In fact the same could be true of bovine XO, being repeatedly "administered" from the diet.

Most human anti-XO antibodies are IgG and have not been shown to be elevated in MI patients. IgM anti-XO antibodies are, however, raised following MI and the most important question concerns their origin. Their levels were shown most clearly to be raised in serum taken from patients within 24h of MI. This is certainly a faster response than would be expected if the antibodies arose from endogenous XO, released in the infarct itself. It may be that the response reflects a pre-clinical event prior to the infarct proper and careful studies of antibody levels in serial samples, following MI, might shed some light on this. If the IgM response was a consequence of the MI or a preceding event, a subsequent rise in IgG anti-XO antibodies might be expected. This was certainly not evident in the "new" (2-3 weeks post MI) samples although it may be that any such rise could be missed against a high background level of exogenously-induced anti-XO antibodies.

A further question arises in connection with the idea that XO, newly exposed in MI, initiates production of IgM antibodies. This question concerns the occurrance of XO in humans. XO has been known to occur in human liver

and intestine, and was shown by Jarasch et al (1981) to be present in capillary endothelial cells, but not arteries, of cows. If the same were true of humans, it could well be that infarct-associated damage in the myocardium would expose endogenous XO to the immune system. It happens that the presence of XO in human heart is currently hotly debated in connection with its proposed role as the initiator of free radical damage following an MI. Most recent opinion (Downey et al,1988) is largely against its significant presence in the human heart. Demonstration that IgM anti-XO antibodies arise in response to myocardial XO would clearly contradict this opinion; conversely, confirmation of the absence of XO in human heart would argue against the idea that the IgM response is secondary to MI.

What experiments could help to answer these many uncertainties? Assays of IgG and IgM anti-XO antibodies in serial serum samples, taken from patients in the days, weeks and months following an MI, may well help to resolve the question of whether the IgM response is secondary to MI. The purification and study of human XO antibodies, also might help answer this question. Human and bovine XO could be used in competition ELISAs, to determine the specificities of both IgM and IgG concerning the possibility that MI is initiated by XO-induced endothelial damage, a search for circulatory immune complexes containing XO might prove profitable.

In conclusion, this thesis has served to resolve many of the apparent contradictions surrounding Davies' findings and hypothesis of MI and may even have gone some way toward validating the ideas of Oster. However very many questions remain and perhaps this work will help to stimulate their solution.

APPENDIX

Although it has been possible to show an elevation of IgM anti-BMFGM antibodies, and more specifically IgM anti-XO antibodies in MI patient compared to controls, this does not exclude the possibility that the observed elevation of IgM is in fact due to rheumatoid factor in MI patients. Rheumatoid factor is an autoantibody, mainly IgM, which reacts with IgG. Immune complexes arising from such a combination are characteristically found in the joints of sufferers of Rheumatoid Arthritis.

If rheumatoid factor is, in fact, elevated in MI patients this would also explain why, in the ELISA measuring specifically IgG or IgA antibodies to either BMFGM or XO, no difference was found between MI and control samples. However, in the ELISA measuring IgM antibody levels to either BMFGM or XO , any IgG binding to the antigen would of course have an IgM antibody (rheumatoid factor) associated with it. This would be detected as well as any IgM anti-BMFGM or IgM anti-XO antibody.

Reference:-

Chantler, S. and Diment, J.A. (1981) in " Imunoassays for the 80's " (Voller, A., Bartlett, A. and Didwell, D. Eds). 417-430, M.T.P.

REFERENCES

Abd-ElFattah, A.S., Jessen, M.E., Lekven, J., Doherty. N.E., Brunstig. L.A., Wechsler. A.S. (1988). Circulation. 78, (5), 224-235.

Abildgard, C.F. and Simone, J.V. (1967). Seminars in Hematology. 4, 424-452.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1983). "Molecular Biology Of the Cell". Garland Publishing, Inc. New York and London.

Al-Khalidi, U.A.S., Nasrallah, S., Kachadurian, A.K. and Shammea, M.H. (1965). Clin. Chim. Acta. 11, 72-77.

Anderson, C.L. and Looney, R.J. (1986). Immumol. Today. 7, 264-266

Anderson, M., Cawston, T., Cheeseman, G.C. (1974). Biochem. J. 139, 653-660.

Anderson, M. and Cheeseman, G.C. (1971). J. Dairy. Res. 38, 409-417.

Anitschkow, N. and Chalatov, S. (1913). Centralblatt fur Allgemeine Pathologie. 24, 1.

Annand, J.C. (1967). J. Atheroscler. Res. 7, 797-801.

Annand, J.C. (1986). Atherosclerosis. 59, 347-351.

Arfors, K.E., Rutili, G., Svensjo, E., (1979). Acta. Physiol. Scand. (suppl). 463, 93-103.

Ascherson, F.M. (1840). Translation in "Foundations of Colloid Chemistry." by Hatschek, E. (1925). E. Benn Ltd. London.

Aster, R.H., Miscovich, B.H. and Rodey, G.E., (1973). Transplantation. 16, 205-210.

Baillie, M. (1793). "The Morbid Anatomy Of Some Of The Most Important Parts Of The Human Body."

Barber, A.J., and Jamieson, G.A. (1970). J. Biol. Chem. 245, 6357-6365.

Bargmann, W. and Knoop, A. (1959). Z. Zellforsch. Mikrosk. Anat. 49, 344-388.

Barrett-Connor, E. and Khaw, K.T. (1988). Circulation. 78, 539-545.

Basch, J.J., Greenberg, R., Farrell, H.M.Jr. (1985). Biochem. Biophys. Acta. 830, 127-135.

Battelli, M.G. (1980). FEBS Letts. 113, 47-51.

Battersby, B., Sherwood, T., Winocour, P., Virella, G. (1984). J. Clin. Lab. Immunol. 15, 57-61.

Baumgartner, H.R. (1963). Z. Ges. Exp. Med. 137, 227-235.

Beale, D. and Hopley, J. (1986). Biochem. J. 233, 37-40.

Beaumont, J.L. and Beaumont, V. (1978) "Immunological Aspects of Atherosclerosis in "Atherosclerosis Reviews Vol 3" ". (Pasletti, R. and Gotto, A.M. Jr. Eds.) Raven Press, New York.

Begg, T.B., Wade, J.M., and Bronte-Stewart, B. (1966). J. Atheroscler. Res. 6, 303-312.

Benboubetra, M. and Harrison, R. Dept of Biochemistry, University of Bath, Claverton Down, Bath, U.K.

Benditt, E.P. and Benditt, J.M. (1973). Proc. Nat. Acad. Sci. U.S.A. 70, 1753-1756.

Bizzozero, G. (1882). Virchows. Arch. Path. Anat. 90, 261-332.

Bjorkerud, S. and Bondjers, G. (1971). Atherosclerosis. 13, 355-363.

Bjorkerud, S. and Bondjers, G. (1972). Atherosclerosis. 15, 285-300.

Bloom, W. and Fawcett, D.W. (1975). "A textbook of Histology". Saunders, Philadelphia

Bondjers, G. and Bjorkerud, S. (1973). Atherosclerosis. 17, 85-94.

Bowman, W.C. and Rand, M.J. (1980). "Textbook of Pharmacology", Blackwell Scientific Publications, Cambridge.

Boyden, S.J. (1951). J. Exp. Med. 93, 107-120.

Bradford, M.M. (1976). Anal. Biochem. 72, 248-254.

Bradley, D.D., Wingerd, J., Petitti, D.B., Krass, R.M. and Ramcharan, S. (1978). New. Engl. J. Med. 299,1, 17-20.

Brandtzaeg, P. (1974). J. Immunol. 112, 1553-1559.

Briley, S.M. and Eisenthal, R. (1974). Biochem J. 143, 149-152.

Brown, M.S., Faust, J.R., and Goldstein, J.L. (1975). J. Clin. Invest. 55, 783-793.

Brown, M.S. and Goldstein, J.L. (1984). Scientific American. 251 (5), 58-66.

Brown, M.S. and Goldstein, J.L. (1986) Science. 232, 34-47.

Bruder, G., Heid, H.W., Jarasch, E-D. and Mather, I.H. (1983). 23, 218-225.

Bruder, G., Jarasch, E.D. and Heid, H.W. (1984). J. Clin. Invest. 74, 783-794.

Bylock, A., Bondjers, G., Jansson, I., Hansson, H.A., (1979). Acta. Pathol. Microbiol. Scand. 87 (sect A), 201-209.

Caerphilly and Speedwell Collaborative Group. (1984). J. Epidemiol. and Comm. Health. 38, 259-262.

Capron, A., Ameisen, J.C., Joseph, M., Auriault, C., Tonnel, A.B. and Caen, J. (1985). Int. Archs. Allergy. Appl. Immunol. 77, 107-114.

Carr, C.J., Talbot, J.M. and Fisher, K.D. (1975). Contract No. FDA 223-75-2090 (Div. Nutrition, Bureau of Foods), FASEB Life Sciences Research Office, Bethesda.

Carruthers, A. and Melchior, D.L. (1983). Biochemistry. 22, 5797-5807.

Carstairs, K.C. (1965). J. Pathol. Bacteriol. 90, 225-231.

Cartron, J.P. and Nurden, A.T. (1979). Nature. 282 621-623.

Cathcart, M.K., Morel, D.W. and Chisolm, G.M. (1985). J. Leuk. Biol. 38, 341-350.

Challacombe, S.J. and Tomasi, T.B. Jr. (1980). J. Exp. Med. 152, 1459-1462.

Chambers, D.E., Parks, D.A., Patterson, G., Roy, R., McCord, J.M., Yoshida, S., Parmley, L.F. and

Charlat, M.L., O'Neil, P.G., Abernathy, D.R., Egan, J.M., Michael, L.H., Roberts, R. and Bolli, R. (1986). Circulation. 74, 11-19.

Clark, D.A., Schroeder, J.S., Gniepp, R.B., Stinson, E.B., Dore, E., Shumway, N.E. and Harrison, D.C. (1973). Am. J. Pathol. 54, 563-576.

Clark, S.L. (1959). J.Biophys. Biochem. Cytol. 5, 41-50.

Clifford Rose, F.(1977). In "Motor Neurone Disease." Ch 1, pp1-13, (Clifford Rose, Ed). Pitman Press.

Cochrane, C.G. and Hawkins, D. (1968). J. Exp. Med. 127, 137-154.

Cooper, G.W. (1970). In "Regulation of Hematopoiesis." (Gordon, A.S. Ed.) VolII p 1611, Appleton-Century-Crofts, New York.

Copley, A.L. (1979). Folia. Haematol. 106, 732-764.

Cornacoff, T.B., Herbert, L.A., Smead, W.L. and Vanaman, M.E. (1983). J. Clin. Invest. 71, 236-247.

Coudrier, E., Reggio, H. and Louvard, D. (1983). EMBO.J. 2, 469-475.

Crawford, T. and Levene, C.I. (1953). J. Pathol. Bacteriol. 66, 19-23.

Crell, J.F. (1740). Cited by Long, E. in "Atherosclerosis" (1953). (Cowdry, E.V. Ed). MacMillan, New York.

Criqui, M.H., Lee, E.R., Hamburger, R.N., Klauber, M.R. and Coughlin, S.S. (1987). Am. J. Med. 82, 964-968.

Cronkite, E.P. (1958). Brook Haven Symp. in Biol. 10, 96-120.

Cunningham, M.J. and Pasternak, R.C., (1988). Circulation. 77, 964-966.

Cunningham-Rundles, C., Brandeis, W.E., Good, R.A. and Day, N.K. (1978). Proc. Nat. Acad. Sci. U.S.A. 75, 3387-3389.

D'Angelo, V., Villa, S. and Mysliewiec, M. (1978). Thrombosis and Haemostasis, 39, 535-536.

Davies, D.F. (1958). Clin.Sci. 17, 563-573.

Davies, D.F. (1959). Clin.Sci. 18, 263-268.

Davies, D.F. (1969). J. Atheroscler. Res. 10, 253-259.

Davies, D.F. (1984). In "Health Hazards of Milk." pp 201-212, (Freed, D.L.J., Ed), Balliere, Tindall.

Davies, D.F. and Clark, A. (1961). Clin.Sci. 20, 279-287.

Davies, D.F. and Clark, A. (1968). J. Atheroscler. Res. 8, 724-730.

Davies, D.F., Davies, J.R. and Richards, M.A. (1969). J. Atheroscler. Res. 9, 103-107.

Davies, D.F., Johnson, A.P., Rees, B.W.G., Elwood, P.C. and Abernethy, M. (1974). Lancet. i, 1012-1014.

Davies, D.F. and Lloyd, V. (1967). Cardiovasc. Res. 1, 108-110.

Davies, D.F., Rees, B.W.G. and Davies, P.T.G. (1980). Lancet. i, 1190-1191.

Davies, D.F., Rees, B.W.G. and Davies, P.T.G. (1982). International Laboratory. 12, 22-24.

Dawber, T.R. (1980) "The Framingham Study: An epidemiological investigation of cardiovascular disease." Harvard. Univ. Press, Cambridge, Mass.

Dawson, R.M.C., Elliot, D.C, Elliot, W.H. and Jones, K.M. (1974). In "Data for Biochemical Research." Oxford Univ. Press.

Deeth, H.C. (1983). J. Dairy. Sci. 66, 1419-1435.

DeScheerder, I., Vandekerckhove, J., Robbrecht, J., Algoed, L., De Buyzere, M., DeLanghe, J., DeSchrijver, G. and Clement, D. (1985). Am. J.Cardiol. 56, 631-633.

Digby, J., Harrison, R., Jehanli, A., Lunt, G.G. and Clifford-Rose, F. (1985). Muscle Nerve 8, 595-605.

Dowben, R.M., Brunner, J.R. and Philpott, D.E. (1967). Biochem. Biophys. Acta. 135, 1-10.

Downey, J.M., Hearse, D.J. and Yellon, D.M. (1988). J. Mol. Cell. Cardiol. 20 (suppl), 55-63.

Duguid, J.B. (1946). J. Pathol. Bacteriol. 58, 207-212.

Duguid, J.B. (1948). J. Pathol. Bacteriol. 60, 57-61.

Eastham, E.J., Lichauco, T., Grady, M.I. and Walker, W.A. (1978). J. Pediatr. 93, 561-564.

Ebringer, A., Rosenbaum, M., Pincus, N. and Doyle, A.E. (1971). Am. J. Med. 50, 297-301.

Eddy, L.J., Stewart, J.R., Jones, H.P., Engerson, T.D., McCord, J.M., Downey, J.M., Cunningham-Rundles, C., Brandeis, W.F. and Good, R.A. (1987). Proc. Nat. Acad. Sci. U.S.A. 75, 3387-3389.

Egger, J., Carter, C.M., Wilson, J., Turner, M.W. and Soothill, J.F. (1983). Lancet ii, 865-869.

Ehrenfeld, E.N., Gery, I. and Davies, A.M. (1961). Lancet. i, 1138-1141.

Eisenberg, M.S., Bergner, L., Hallstrom, A.P. and Cummings, R.O. (1986). Scientific American. 254, 25-31.

Ellis, F.R. (1974). Lancet. ii, 400.

Elwood, P.C., Beswick, A. and Cartwright, J. (1988). Br. J. Haematol. 34 (suppl) 45 (abstract).

Engerson, T.D., McKelvey, T.G., Rhyne, D.B., Boggio, E.B., Snyder, S.J. and Jones, H.P. (1987). J. Clin. Invest. 79, 1564-1570.

Engle, M.A. and Ito, T. (1961). Am. J. Cardiol. 7, 73-82.

Faggiotto, A. and Ross, R. (1984). Arteriosclerosis. 4, 341-356.

Faggiotto, A., Ross, R. and Harker, L. (1984). Arteriosclerosis. 4, 323-340.

Ferguson, A. (1977). Gut. 18, 921-937.

Finkelstein, A.E., Woerner, T.E., Smith, J.C., Bayles, T.B. and Levine, H.D. (1963). Am J. Med. 35, 163-174.

Fisher, R.B. (1954). In "Protein Metabolism." Methuen, London.

Florey, H. (1960). Br. Med. J. 2, 1329-1335.

Forrester, J.S., Litvack, F., Grundfest, W. and Hickey, A. (1987). Circulation. 75, 505-513.

Franke, W.W., Heid, H.W., Grund, C., Winter, S., Freudenstein, C., Schmid, E., Jarasch, E.D., Keenan, T.W., (1981), J. Cell. Biol. 89, 485-494.

Freifelder, D. (1982). In "Physical Biochemistry".p688. W.H. Freeman and Co, New York.

Fust, G., Szondy, E., Szekely, J., Nanai, I. and Gero, S. (1978). Atherosclerosis. 29, 181-190.

Gajdusek, C.M., Dicorleto, P., Ross, R. and Schwartz, S.M. (1980). J. Cell. Biol. 85, 467-472.

George, J.N., Nurden, A.T. and Phillips, D.R. (1984). New. Eng. J. Med. 311, 1084-1098.

George, J.N., Nurden, A.T. and Phillips, D.R. (1985). "Platelet Membrane Glycoproteins." Plenum Press, New York.

George, J.N. and Saucerman, S. (1988). Blood. 72, 362-365.

Gerrity, R.G. (1981). Am J. Pathol. 103, 181-200.

Gibney, M.J., Gallagher, P.J., Sharratt, G.P., Benning, H.S., Taylor, T.G. and Pitts, J.M. (1980). Atherosclerosis. 37, 151-155.

Giler, S., Sperling, O., Brosh, S., Urca, I. and DeVries ,A. (1975). Clin. Chim. Acta. 63, 37-40.

Gordon, D.J., Probstfield, J.L., Garrison, R.J., Neaton, J.D., Castelli, W.P., Knoke, J.D., Jacobs, D.R., Bangdiwala, S. and Tyroler, H.A. (1989). Circulation. 79 (1), 8-15.

Gorlin, R., Fuster, V. and Ambrose, J.A. (1986). Circulation. 74, 6-9.

Granger, D.N., Rutili, G. and McCord, J.M. (1981). Gastroenterology. 81, 22-29.

Gray, M.V., Hill, J.D., and Mitchell, J.R.A. (1978). Atherosclerosis, 31, 377-381.

Greenbank, G.R. and Pallansch, M.J. (1962). J. Dairy. Sci. 45, 958-961.

Greenwalt, D.E., Johnson, V.G. and Mather, I.H. (1985). Biochem. J. 228, 233-240.

Gregoriardis, G. (1976). New. Eng. J. Med. 295, 704-710.

Guilbert, B., Dighiero, G. and Avrameas, S. (1982). J Immunol. 128, 2779-2787.

Gunther, M., Aschaffenburg, R., Matthews, R.H., Parish, W.E. and Coombs, R.R.A. (1960). Immunology. 3, 296-306.

Habeeb, A.F.S.A. and Hiramoto, R. (1968). Arch. Biochem. Biophys. 126, 16-26.

Haerem, J.W. (1975). Am. Heart. J. 90, 562-568.

Hamburger, F. (1901). "Biologisches uber die Eiweeisskerperder kuhmilch und uber sauglingsnahrung wien klin wochen schr", 14, 1202.

Hamburger, R.N., Casillas, R., Johnson, R., Mellon, M., O'Conner, R.D. and Zeiger, R. (1987). Annals of Allergy. 59 (2), 175-178.

Hamsten, A., Norberg, R., Bjorkholm, M., De Faire, U. and Holm, G. (1986). Lancet. i, 113-115.

Hancock, K. and Tsang, V.C.W. (1983). Anal. Biochem. 133, 157-162.

Hannson, G.K. and Bjornheden, T., Bylock, A. and Bondjers, G. (1981). Exp. Mol. Pathol. 34, 264-280.

Hannson, G.K., Bondjers, G., Bylock, A. and Hjalmarsson, L. (1980). Exp. Mol. Pathol. 33, 302-315.

Hannson, G.K., Starkenbaum, G.A., Benditt, E.P. and Schwartz, S.M. (1984). Proc Nat Acad Sci, U.S.A. 81, 3103-3107.

Harkavy, J. and Perlman, E. (1964). N.Y. State. J. Med. 64, 1287-1296.

Harrison, R. and Behan, P.O. (1986) in "Clinical Neurochemistry." vol 1. (Bachelard, H., Lunt, G.G. and Marsden. D., Eds). Acad. Press.

Haust, M.D. (1971). Human Pathology. 2, 1-30.

Hayslett, H.T. (1976). In "Statistics Made Simple". (Murphy, P. Ed). Chaucer Press, Bungay.

Hearse, D.J., Manning, A.S., Downey, J.M. and Yellon, D.M. (1986). Acta. Physiol. Scand. 126, 65-78.

Heine, W.I., Friedman, H., Mandell, M.S. and Goldberg, H. (1966). Am. J. Cardiol. 17, 798-803.

Heird, W.C. and Hansen, I.H. (1977). Pediatr. Res. 11, 406.

Heller, T., Bailey, L., Gott, M. and Howes, M. (1987) In "Coronary Heart Disease - reducing the risk". John Wiley and sons Ltd, Chichester.

Hemmings, W.A. (1978). In "Antigen Absorption by the Gut. "MTP Press Ltd, Lancaster.

Henson, P.M. and Spielberg, H.L. (1973). J. Clin. Invest. 52, 1282-1288.

Hodgson, J. (1815). In "Treatise on the diseases of arteries and veins."

Hopkins, P.N. and Williams, R.R. (1981). Atherosclerosis. 40, 1-52.

Horai, S., Claas, F.H.J. and van Rood, J.J. (1981) Immunol. Letts. 3, 67-72.

Huang, C.M. and Keenan, T.W. (1972). Biochem.Biophys. Acta. 274, 246-257.

Huang, R.T.C. (1973). Biochem. Biophys. Acta. 306, 82-84.

Hudson, L. and Hay, F.C. (1976) "Practical Immunolgy." Blackwell Scientific Publications, Oxford.

Hutchins, P. and Walker-Smith, J.A. (1982). In "Clinics in Immunology and Allergy." Vol2, No 1 pp43-77.

Israels, E.D., Wisli, G., Paraskevas, F. and Israels, L.G. (1973) Thromb. Diath. Haemorr. 29, 434-444.

Iverius, P.H. (1973). In " Atherogenesis:Initiating Factors." CIBA Symp. No 12, 185-196, Elsevier.

Jackson, R.L. and Gotto, A.M.Jr. (1976). In Atherosclerosis Reviews." Vol 1, pp 1-21, (Paoletti, R. and Gotto, A.M. Jr, Eds). Raven Press, New York.

Jaffe, E.A., Hoyer, L.W. and Nachman, R.L. (1973). Proc. Nat. Acad. Sci. U.S.A. 71, 1906-1909.

Jarasch, E.D., Bruder, G. and Heid, H.W. (1986). Acta Physiol. Scand. suppl. 548, 39-46.

Jarasch, E.D., Bruder, G., Keenan, T.W. and Franke W.W. (1977). J. Cell. Biol. 73, 223-241.

Jarasch, e.d., Grund, C., Bruder, G., Heid, H.W., Keenan, T.W. and Franke, W.W. (1981). Cell. 25, 67-82.

Jenkins, P.J., Harper,.R.W. and Nestel, P.J. (1978). Br. Med. J. ii, 388-391.

Jenner, E. (1778). in "Dr Jenner of Berkeley:Letter to Heberden", p68, (Fisk, D. Ed). Heinneman, London.

Jewell D.P. and Trulelove, S.C. (1972). Gut. 13, 796-801.

Joel, D.D., Sordat, B., Hess, M.W. and Cottier, H. (1970). Experentia. 26, 694 (Abstract).

Johnson, S.A. (1971). In "The Circulating Platelet" (Johnson, S.A. Ed). Academic Press, New York.

Johnson, D.A., Gautch, J.W., Sportsman, J.R. and Elder, J. (1984). Gene. Anal. Tech. 1, 3-8.

Johnson, V.G., Greenwalt, D.E., Heid, H.W., Mather, I.H. and Nadara, P.J. (1985). Eur. J. Biochem. 151, 237-244.

Johnstone, A. and Thorpe, R. (1982). "Immunochemistry in Practice." Blackwell Scientific Publications, Oxford.

Kabat, E.A. (1968). "Structural Concepts in Immunology and Immunochemistry". Holt, Rinehart and Winston Inc, New York.

Karas, S.P., Rosse, W.F. and Kurlander, R.J. (1982). Blood. 60, 1277-1282.

Kaser-Glanzmann, R., Jakabova, M., George, J.N. and Luscher, E.F. (1977). Biochem. Biophys. Acta. 466, 429-440.

Kay, M.M.B. (1981). Nature. 281, 491-494.

Keenan, T.W. (1974). Biochem. Biophys. Acta. 337, 255-270.

Keenan, T.W. and Huang, C.M. (1972). J. Dairy. Sci. 55, 1586-1596.

Keenan, T.W., Morre, D.J., Olson, D.E., Yunghans, W.N. and Patton, S. (1970). J. Cell. Biol. 44, 80-93.

Kelton, J.G. and Gibbons, S. (1982). Semin. Thromb. Haemost. 8, 83-104.

Kennel, W.B., Hjortland, M.C., McNamara, P.M. and Gordon, T. (1976). Ann. Inerm. Med. 85, 447-452.

Keys, A. (1970). Circulation. 41 Suppl I, II-I211.

Keys, A. (1980). "Seven Countries: a multivariate analysis of death and CHD". Harvard Univ. Press, Cambridge, Mass.

King, N. (1955). Tech. Commun. No2, of Commonwealth Bureau of Dairy Science, pp1-99.

Kinsman, J.M., Murray, C.E., Richard, V.J., Jenning, R.B. and Reimer, K.A. (1988). J. Am. Coll. Cardiol. 12, 209-217.

Kirkwood, E. and Lewis, C. (1989). In "Understanding Medical Immunology". John Wiley and Sons Ltd, London.

Kobylka, D. and Carraway, K.L. (1973). Biochem. Biophys. Acta. 307, 133-140.

Korenblat, P.E., Rothberg, R.M., Minden, P. and Farr, R.S. (1968). J. Allergy. 41, 226-235.

Kuhn, L.C. and Kraehenbuhl, J-P. (1979). J. Biol. Chem. 254, 11066-11071.

Laegreid, A., Otnaess, A.K. and Fuglesang, J. (1986). Pediatric. Res. 20, 416-421.

Laemmli, U.K. (1970). Nature. 227, 680-685.

Lang, B., Vincent, A. and Newsom-Davies, J. (1982). J. Immun. Meth. 51, 371-381.

LeFevre, M.E. and Joel, D.D. (1984). "Intestinal Toxicology." pp45-56. (Schiller, C.M. Ed), Raven Press, New York.

Leibovich, S.J. and Ross, R. (1976). Am. J. Pathol. 84, 501-514.

Lev, R. and Orlic, D. (1973). Gastroenterology. 65, 60-68.

Levine, R.F. (1982). Br J. Haematol. 52, 173-180.

Levy, L. (1967). In "Advances in Exp. Med. and Biology: The reticuloendothelial system and atherosclerosis." Vol 1. (DiLuzio, N.R. and Paoletti, R. Eds). Plenum Press, New York.

Lin, W. and Kasamatsu, H. (1983). Anal. Biochem.128, 302-311.

Linder, E. (1981). J. Immunol. 126, 648-658.

Link, E.M. and Riley, P.A. (1980). Biochem. J. 249, 391-399.

Lippard, V.W., Schloss, O.M. and Johnson, P.A.(1936). Am. J. Dis. Child. 51, 562-574.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). J. Biol. Chem. 193, 265-275.

L.R.C.P. (The Lipid Research Clinics Program) (1984). J. Am. Med. Assoc. 251, 351-364.

MacDonald, V.E. and Savoca, K.V. (1985). Int. Clin. Prod. Rev. (Jan/Feb), 8-18.

McCarthy, R.D. and Long, C.A. (1976). J. Dairy. Sci. 59, 1059-1062.

McCord, J.M. (1985). New. Engl. J.Med. 312, 159-162.

McDonald, L. (1960). Proc. Roy. Soc. Med. 53, 35-38.

McGill, H.C.Jr. (1984). Arteriosclerosis. 4, 443-451.

Marchand, F. (1904). Ver handlung der Kongres fur innere Medizin. 21, 23-59.

Markwell, M.A.K., Haas, S.M., Tolbert, N.E. and Bieber, L.L. (1981). Meth. in. Enzymol. 72, 296-303.

Matel, M.B., Dubois, P. and Got, R. (1973). Biochem. Biophys. Acta. 311, 565-575.

Mather, I.H. (1978). Biochem. Biophys. Acta. 514, 25-36.

Mather, I.H. and Keenan, T.W. (1975). J.Memb. Biol. 21, 65-85.

Mather, I.H., Sullivan, C.H. and Madara, P.J.(1982), 202, 317-323.

Medof, M.E., Iida, K., Mold, C. and Nussenzweig, V. (1982). J. Exp. Med. 156, 1739-1754.

Minick, C.R. (1976). Ann. New. York. Acad. Sci. 275, 210-226.

Minick, C.R. and Murphy, G.E. (1973). Am. J. Pathol. 73, 265-300.

Mitchell, J.R.A. (1978). British Medical Bulletin. 34, 105-106.

Mitchell, J.R.A. and Schwartz, C.J. (1965). In "Arterial Disease", pp50-65, (Davis, F.A. Ed). Blackwell Scientific, Philadelphia.

Monacada, S., Gryglewski, R.J., Bunting, S. and Vane, J.R. (1976). Nature. 261, 663-665.

Monro, J., Carini, C. and Brostoff, J. (1984). Lancet. ii, 719-721.

Moore, S. (1974). "Vascular Injury and Atherosclerosis". Marcel Dekker Inc, New York.

Moore, S., Friedman, R.J., Singal, D.P., Blajchman, M.A. and Roberts, R.S. (1976). Thromb. Haemost. 35, 70-81.

Moreau, P. and Andre, A. (1954). Nature. 174, 88-90

Morrisey, J.H. (1981). Anal. Biochem. 117, 307-310.

Morton, R.K. (1954), Biochem. J. 57, 231-237.

Mostov, K.E., Friedlander, M. and Blobel, G. (1984). Nature. 308, 37-43.

Mowat, A.M. (1987). Immunology Today. 8 (3), 93-98.

Moxey, P.C. and Thier, J.S. (1975). Gastroenterology. 68, 1002-1009.

Murano, G. and Bick, R.L. (1980). In "Basic Concepts of Hemostasis and Thrombosis: Clinical and laboratory evaluation of thrombohemorrhagic phenomena." CRC Press Inc, Boca Raton.

Nachman, R.L. and Weksler, B. (1980) in "The Cell Biology of Inflammation" (Weissman, G. Ed). 145-160, Elsevier, Amsterdam.

Nagler, L.G. and Vartanyan, L.S. (1976). Biochem. Biophys. Acta. 427, 78-90.

Nakamura, M. and Yamazaki, I. (1982). J.Biochem. 92, 1279-1286.

Newman, R.A. and Harrison, R. (1973). Biochem. Biophys. Acta. 298, 798-809.

Newsom-Davies, J. and Vincent, A. (1982). in "Clinical Aspects of Immunology" 4th edn, Vol2 pp1011 1068 Lachmann, P.J. and Peters, D.K. Eds). Blackwell Scientific Publications.

Nielsen, C.S. and Bjerrum, O.J. (1977). Biochem. Biophys. Acta. 446, 496-509.

Nishino, Y., Nishino, T. and Tsushima.K (1981). FEBS. Lett. 131, 369-372.

Oliver, M.F. and Boyd, G.S. (1959). Lancet. ii, 690-694.

O.P.C.S. Monitor (1982). "Death by cause (DH2) 1982". Public office of population censuses and surveys, London.

Oster, K.A. (1968). Cardiol. Digest. 3, 29-34.

Oster, K.A. (1971). Am J. Clin. Res. 2, 30-35.

Oster. K.A. (1972). In "Myocardiology" Vol 1.,pp 803-813, (Bajusz, E. and Rona, G. Eds). Univ. Park. Press, Baltimore.

Oster, K.A. (1973a). in "Myocardial Metabolism." pp 73-80, (Dhalla, N.S. Ed). Univ. Park. Press, Baltimore.

Oster, K.A. (1973b). Medical Counterpoint. 5, 26-36.

Oster, K.A. (1976). Am. Lab. 8, 47-49.

Oster, K.A. and Hope-Ross, P. (1966). Am. J. Cardiol. 17, 83-85.

Oster, K.A. and Mulinos, M.G. (1944). Pharmacol. Exp. Theor. 84, 132-138.

Oster, K.A., Oster, J.B. and Ross, D.J. (1974). Am. Lab. 6, 41-47.

Oster, K.A. and Ross, D.J. (1975). Recent. Adv. Stud. Cardiac. Struct. Metab. 10, 401-410.

Owen, R.L. (1977). Gastroenterology, 72, 440-451.

Paganelli, R., Levinsky, R.J., Brostoff, J., Wraith, D.C. (1979) Lancet. i, 1270-1272.

Page, C.P. (1988). Trends in Pharm. Sci. 9, 66-71.

Palmer, L.S. and Samuelson, E. (1924). Proc. Soc. Exp. Biol. Med. 21, 537-539.

Parish, W.E., Barrett, A.M., Coombs, R.R.A., Camps, F.E. and Gunther, M. (1960). Lancet. ii, 1106-1110.

Parker, R.E. (1973). In "Introductory Statistics for Biology". Edward Arnold Ltd, London.

Parks, D.A. and Granger, D.N. (1986). Acta. Physiol. Scand. Suppl. 548, 87-99.

Patton, S. and Keenan, T.W. (1975). Biochem. Biophys. Acta. 415, 273-309.

Patton, S. and Trams, E.G. (1971). Febs. Lett. 14, 230-232.

Pfueller, S.L. and Luscher, E.F. (1972). J. Immunol. 109, 517-525.

Phillips, D.R. and Agin, D.P. (1977). Biochem. Biophys. Res. Commun. 75, 940-947.

Playfair, J.H.L. (1982). "Immunology at a Glance". Blackwell Scientific Publications, Oxford.

Poole, J.C.F., Sanders, A.G. and Florey, H.W. (1958). J. Pathol. Bact. 75, 133-158.

Poston, R.N. (1975). Lancet. ii, 502-503.

Poston, R.N. and Davies, D.F. (1974). Atherosclerosis. 19, 353-367.

Powell, J.T., Jarlfors, U. and Brew. K. (1977). J. Cell. Biol. 72, 617-627.

Puett, D.W., Forman, M.B., Cates, C.V., Wilson, B.H., Hande, K.R., Friesinger, G.C., Virmani, R. (1987). Circulation. 76, 678-686.

Raz, A.M., Minkes, M.S. and Needleman, P. (1977) Biochem. Biophys. Acta. 488, 305-311.

R.C.G.P. (Royal College of General Practitioners) (1986). "Morbidity statistics from general practice 1981-1982. Third national survey" HMSO, London.

Rees, B.W.G. (1973). Med. Lab. Tech. 30, 167-177.

Rees, B.W.G. (1985). PhD Thesis."The Interaction of Platelets and Exogenous Antigenic Factors in Coronary Heart Disease." Open University.

Rees, B.W.G. Dept of Haematology, West Wales Hospital, Carmarthen, Wales, U.K.

Reid, J., Research and Development Division, Flow Lab, Second Avenue, Industrial Estate, Irvine, Scotland U.K.

Reidy, M.A. (1985). Lab. Invest. 53, 513-520.

Reidy, M.A. (1986). In "Biology and Pathology of Platelet Vessel Wall Interactions." (Jolles. G., Legrand, Y.J. and Nurden.A. Eds). Proc. Rhone Poulenc Sante Inserm conference. Academic Press, London.

Reidy, M.A. and Schwartz, S.M. (1984). Exp. Mol. Pathol. 41, 419-434.

Reimer, K.A. and Jennings, R.B. (1985). Circulation. 71, 1069-1075.

Reinhardt, M.C., Paganelli, M.D. and Levinsky, R.J. (1983). Ann. Allergy. 51, 311-312.

Rhodes, R.S. and Karnovsky, M.J. (1971). Lab. Invest. 25, 220-229.

Richards, F.M. and Knowles, J.R. (1968), J. Mol. Biol. 37, 231-233.

Richman, L.K., Graef, F.A.S., Yarchsan, R. and Shober, W. (1981). J.Immunol. 126, 2079-2083.

Robson, C. (1973). In "Experiment, Design and Statistics in Psychology". (Foss, B.M. Ed). Chaucer Press, Bungay.

Roitt, I. (1977). "Essential Immunology". Blackwell Scientific Publications.

Roitt, I. (1988). "Essential Immunolgy". Blackwell Scientific Publications.

Roitt, I., Brostoff, J. and Male, D. (1985). "Immunolgy". Gower Medical Publishing C, New York and London.

Rosenberg, R.D., Reilly.C. and Fritze, L. (1985). Ann. New York. Acad. Sci. 454, 270-278.

Ross, D.J., Ptaszynski, M. and Oster, K.A. (1973). Proc. Soc. Exp. Biol. Med. 144, 523-526.

Ross, P.J., Sharnick, S.V. and Oster, K.A. (1980). Proc. Soc. Exp. Biol. Med. 163, 141-146.

Ross, R. (1986). New. Eng. J. Med. 314, 488-500.

Ross, R., Glomset, J., Kariya, B. and Harker, L.A (1974). Proc. Nat. Acad. Sci. U.S.A. 71, 1207-1210.

Rothberg, R.M. (1969)· J. Pediatr. 75, 391-399.

Rudel, L.L., Bond, M.G. and Bullock, B.C. (1985). Ann. New York. Acad. Sci. 454, 248-253.

Ruttenberg, E.J., Steerenberg, P.A., Brosi, B.J.M. and Buys, J. (1976). J. Imm. Meth. 10, 67-83.

Rutter, J.V., Harrison, R., Jehanli. A. and Lunt, G.G. (1986). Biochem. Soc. Trans. 1236, 619th meeting. Cambridge, U.K.

Rzucidlo, S.J. and Zikakis, J.P. (1979). Proc. Soc. Exp. Biol. and Med. 160, 477-482.

Saphir, O. and Gore, I. (1950). Arch. Pathol. 49, 418-425.

Schloss, O.M. (1924-1925). Harvey Lectures. 10, 156-187.

Schwartz, S.M. and Benditt, E.P. (1976). Proc. Nat. Acad. Sci. 73, 651-653.

Scott, B.B., McGuffin, P., Swinburne, M.L. and Losowsky, M.S.(1976). Lancet. ii, 125-126.

Seely, S. (1981). Medical Hypoth. 7, 907-918.

Segall, J.J. (1977). Brit. J. Prev. Soc. Med. 31, 81-85.

Seifert R.A., Schwartz, S.M. and Bowen-Pope, D.F. (1984). Nature. 311, 669-671.

Sela, B. and Edelman, G.M. (1977). J. Exp. Med. 145, 443-449.

Shamma'a, M.H., Nasrallah, S.M. and Al-Khalidi, U.A.S. (1973). Dig. Dis. 18, 15-22.

Shaper, A.G., Pocock. S.J., Walker, M., Cohen, N.M., Wale, C.J. and Thompson, A.G. (1981). Brit. Med. J. 2, 179-186.

Shimamoto, T. (1963). J. Athero. Res. 3, 87-102.

Shimamoto, T. and Numano, F. (1969). In "Atherogenesis" (International Congress series, No 201). Excerpta Medica, Amsterdam.

Shimokado, K., Raines, E.W., Madtes, D.K., Barret, T.B., Benditt, E.P. and Ross, R. (1985). Cell. 43, 277-286.

Shekelle, R.B., Shryock, A.M., Oglesby, P., Lepper, M., Stamler, J., Liu, S.and Raynor, W.J. (1981). New. Eng. J. Med. 304, 65-70.

Shiner, M. and Ballard, J. (1972) Lancet. i, 1202-1205.

Shulman, N.R. (1964). Ann. Intern. Med. 60, 507-521.

Siegel, S. (1956). In "Nonparametric statistics For the Behavioural Sciences". McGraw Hill Book Co. Inc, New York.

Silk, D.B.A. (1974). Gut. 15, 494-495.

Smith, G.W., McArthur, C.J. and Simpson, I.J. (1983). J. Clin. Lab. Immunol. 12, 197-199.

Sniderman, A., Shapiro, S., Marpole, D., Skinner, B., Teng, B. and Kwiterovich, P.O. Jr. (1980). Proc. Nat. Acad. Sci. U.S.A. 77, 604-608.

Stamler, J., (1963). In "Atherosclerosis and its origin", pp 231-262. (Sandler, M.and Bourne,G.H. Eds). Acad. Press, New York.

Steiner, M. (1985). Biochem. Biophys. Res. Commun. 129, 206-212.

Stryer, L. (1981). "Biochemistry". W.H.Freeman and Co, San Francisco.

Switzer, R.C., Merril, C,R. and Shifrin, S. (1979). Anal. Biochem. 98, 238-241.

Szczeklik, A., Sladek, K., Szczerba, A. and Dropinski, J. (1988). Circulation. 77, 1245-1249.

Tangen, O., Berman, H.J. and Marfey, P. (1971). Thromb. Diath. Haemorrh. 25, 268-278.

Taussig, M.J. (1979). In "Processes in Pathology", Blackwell Scientific Publications.

Taylor, K.B. and Truelove, S.C. (1961). Br. Med. J. 2, 924-929.

Thiery, J.P. and Bessis, M. (1956). Rev. Hemat. 1, 162-175.

Thoma, R. (1883). Virchows. Archiv. 104, 209.

Thomas, E. Dept of Haematology, West Wales Hospital, Carmarthen, Wales, U.K.

Thompson, C.B., Eaton, K.A., Princiotta, S.M., Rushin, C.A. and Valeri, C.R. (1982). Br. J. Haematol. 50, 509-519.

Thompson, J.G. (1969). Lancet. ii, 1088-1092.

Thompson, M.P., Brunner, J.R., Lindqvist, K. and Stine, C.M. (1961). J. Dairy. Sci. 44, 1589-1596.

Tofler, G.H., Brelinska. and Schafer, A.I. (1987). New. Eng. J. Med. 316, 1514-1518.

Toivanen, A., Vilijanen, M.K. and Savilahti, E. (1975a). Lancet. ii, 205-207.

Toivanen, A., Vilijanen, M.K. and Savilahti, E. (1975b). Lancet. ii, 927.

Towbin, H. and Gordon, J. (1984). J. Immunol. Meth. 72, 313-340.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Proc. Nat. Acad. Sci. U.S.A. 76, 4350-4354.

Trent, R., Clancy, R., Danis, V. and Basten, A. (1980). Br. J. Haematol. 44, 645-654.

Triolo, G., Traina, M., Giardina, E., Rinaldi, A. and Raineri, A. (1984). J. Clin. Lab. Immunol. 13 (1), 35-39.

Uhlenhuth, G. (1900). Deutsche. Med. Woch. 26, 734.

Ultmann, J.E., Feigelson, P. and Harris, S. (1962). J.Immunol. 88, 113-120.

Underdown, B.J. and Schiff, J.M. (1986). In "Annual Review of Immunology". pp389-417.(Paul. W.E., Fathman, C.G. and Metzger, H. Eds), Annual Reviews Inc. California.

Van Es, L.A., Daha, M.R., Valentijn, R.M. and Kauffman, R.H. (1984) Neth. J. Med. 27, 350-358.

Van Leeuenhoek, A. (1674). Phil. Trans. 9, 102.

Van Leeuwen, E., Von Dem Borne, A., Van Der Plas Van Dalen, C. and Engelfriet, C. (1981). Scand. J. Haematol. 26, 285-291.

Vemura, K., and Pisa, Z. (1985). World Health Statistics Quarterly. 38, 142-162.

Vincent, A. (1979). J. Clin. Path. 13, 97-106.

Vines, G. (1989). New Scientist. No 1653, 44-49.

Virchow, R. (1856). In "Phlogose und Thrombose im Gefass-system Gesammelte Abhandlungen zur wissenschaftlichen Medizin" p458. Meidinger, Frankfurt.

Vogel, J. (1847). In " the Pathological Anatomy of the Human Body". Cited By T. Crawford. (1955). Lean and Blanchard, Philadelphia.

Volp, R.F. and Lage, G.L. (1977). Proc. Soc. Exp. Biol. Med. 154, 488-492.

Von dem Borne, A.E.G.K., Van Leeuwen, E.F., Von Riesz, L.E., Van Boxtel, C.J. and Engelfriet, C.P. (1981). Blood. 57, 649-656.

Von Haller, A. (1755). Opuscula Pathologica Observatio, L1.

Von Rokitansky, C. (1844). In "Handbuch der PathologischenAnatomie" Vol 2. Braunmuller and Seidel, Vienna.

Walker, W.A. (1975). Pediatr. Clin. North. Am. 22, 731-746.

Walker, W.A. (1978). Arch. Dis. Chid. 53, 527-531.

Walker, W.A., (1979). Ciba. Found. Symp. 70, 201-219.

Walker, W.A. (1985). Pediatrics (suppl). 75, 167-171.

Walker, W.A. and Isselbacher, K.J. (1974). Gastroenterology. 67, 531-550.

Walker, W.A. and Isselbacher, K.J. (1977). New Eng. J. Med. 297, 767-773.

Walsh, C., Nardi, M. and Karpatkin, S. (1984). New. Eng. J. Med. 311, 635-639.

Walstra, P. (1966). Neth. milk. Dairy. J. 23, 99-110.

Walstra, P. (1974). Neth. milk. Dairy. J. 28, 3-9.

Warburg, O. and Christian, W. (1940). Biochem. J. 303, 40.

Warsaw, A.L., Walker, W.A. and Isselbacher, K.J. (1974). Gastroenterology. 66, 987-995

Weiler, I.J., Hickler, W., Sprenger, R. (1983). Am J. Repro Immunol. 4, 95-98.

Weisgraber, K.H., Rall. S.C. Jr, Mahley, R. W., Milne, R.W., Marcel, Y.L. and Sparrow, J.T. (1986). J. Biol. Chem. 261, 2068-2076.

Weiss, H.J., Hoyer, L.W., Pickles, F.R., Varna, A. and Rogers, J. (1973). J. Clin. Invest. 52, 2708-2716.

Wells, N. (1982). In "Coronary Heart Disease:the scope for prevention". Office of Health Economics, studies of current health problems: no 73, London.

Wells, N. (1986). In "Coronary Heart Disease: The need for action". Office of Health Economics, studies of current health problems; no 86, London.

Werns, S.W., Shea, M.J. and Lucchesi, B.R. (1986). Circulation, 73, 518-524.

Wilson, S.J. and Walzer, N. (1935). Am. J. Dis. Child. 50, 49-57.

Wiman, K., Curman, B., Tragard, R.L., Peterson, P.A. (1979). Eur. J. Immunol. 9 (3), 190-195.

Winiarski, J. and Ekelund, E. (1986). J. Exp. Immunol. 63, 459-465.

Wintrobe (1974). In "Clinical Haematology". Lea and Febiger, Philadelphia.

Wiseman, G. (1964). In "Absorption from the Small Intestine". Academic Press, New York and London.

Wolinsky, H., Goldfischer, S., Daly, M.M., Kasak, L.E. and Coltoff-Schiller, B. (1975). Circ. Res. 36, 553-561.

Wooding, F.B.P. (1971). J. Cell. Sci. 9, 805-821.

Wooding, F.B.P. (1973). J. Cell. Sci. 13, 221-235.

Woolf, N. (1982). In "Pathology of Atherosclerosis". (Crawford, T.Ed). Butterworths and Co Ltd, London and Frome.

Zak, F.G., Halpern, M. and Adlerberg, D. (1952). Angiology. 3, 289.

Zikakis, J.P., Rzucidlo, S.J. and Biasotto, N.O. (1976). J. Dairy. Sci. 60, 533-541.

Zikakis, J.P. and Wooters, S.G. (1980). J. Dairy. Sci. 63, 893-904.

Zittle, C.A., Dellamonica, E.S., Custer, J.H., Rudd, R.K., (1956). J. Dairy. Sci. 39, 528-535.